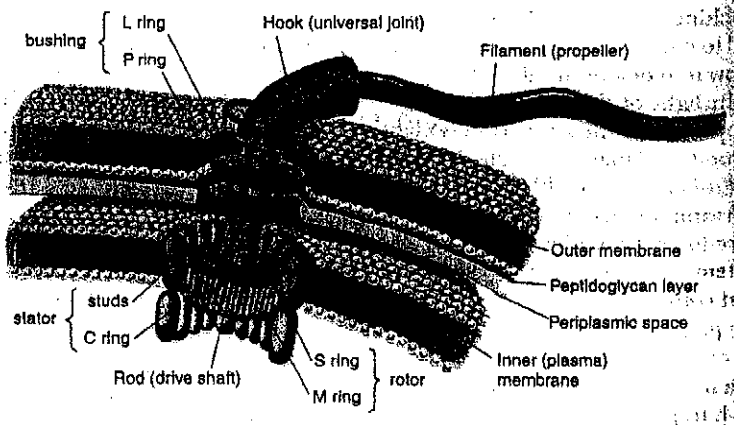


FIGURE 19.1. The bacterial flagellum. Reproduced from D. Voet and J. G. Voet, *Biochemistry*, 2nd ed. (New York: Wiley, 1995), Figure 34–84, with permission of John Wiley Publishers and Donald Voet, who wished to emphasize that "this is an artist-drawn representation of the flagellum rather than a photo or drawing of an actual flagellum."

tail that acts as a propeller; when it is spun, it pushes against the liquid medium and can propel the bacterium forward. The propeller is attached to the drive shaft indirectly through something called the hook region, which acts as a universal joint. The drive shaft is attached to the motor, which uses a flow of acid or sodium ions from the outside to the inside of the cell to power rotation. Just as an outboard motor has to be kept stationary on a motorboat while the propeller turns, there are proteins that act as a stator structure to keep the flagellum in place. Other proteins act as bushings to permit the drive shaft to pass through the bacterial membrane. Studies have shown that thirty to forty proteins are required to produce a functioning flagellum in the cell. About half of the proteins are components of the finished structure, while the others are necessary for the construction of the flagellum. In the absence of almost any of the proteins – in the absence of the parts that act as the propeller, drive shaft, hook, and so forth – no functioning flagellum is built.

As with the mousetrap, it is quite difficult to see how Darwin's gradualistic process of natural selection sifting random mutations could produce the bacterial flagellum, since many pieces are required before its function appears. A hook by itself, or a driveshaft by itself, will not act as a propulsive device. But the situation is actually much worse than it appears from this cursory description, for several reasons. First, there is associated with the functioning of the flagellum an intricate control system, which tells the flagellum ı to rotate, when to stop, and sometimes when to reverse itself



and rotate in the opposite direction. This allows the bacterium to swim toward or away from an appropriate signal, rather than in a random direction that could much more easily take it the wrong way. Thus the problem of accounting for the origin of the flagellum is not limited to the flagellum itself but extends to associated control systems as well.

Second, a more subtle problem is how the parts assemble themselves into a whole. The analogy to an outboard motor fails in one respect: an outboard motor is generally assembled under the direction of a human – an intelligent agent who can specify which parts are attached to which other parts. The information for assembling a bacterial flagellum, however (or, indeed, for assembling any biomolecular machine), resides in the component proteins of the structure itself. Recent work shows that the assembly process for a flagellum is exceedingly elegant and intricate (Yonekura et al. 2000). If that assembly information is absent from the proteins, then no flagellum is produced. Thus, even if we had a hypothetical cell in which proteins homologous to all of the parts of the flagellum were present (perhaps performing jobs other than propulsion) but were missing the information on how to assemble themselves into a flagellum, we would still not get the structure. The problem of irreducibility would remain.

Because of such considerations, I have concluded that Darwinian processes are not promising explanations for many biochemical systems in the cell. Instead, I have noted that, if one looks at the interactions of the components of the flagellum, or cilium, or other irreducibly complex cellular system, they look like they were designed – purposely designed by an intelligent agent. The features of the systems that indicate design are the same ones that stymie Darwinian explanations: the specific interaction of multiple components to accomplish a function that is beyond the individual components. The logical structure of the argument to design is a simple inductive one: whenever we see such highly specific interactions in our everyday world, whether in a mousetrap or elsewhere, we unfailingly find that the systems were intentionally arranged – that they were designed. Now we find systems of similar complexity in the cell. Since no other explanation has successfully addressed them, I argue that we should extend the induction to subsume molecular machines, and hypothesize that they were purposely designed.

## MISCONCEPTIONS ABOUT WHAT A HYPOTHESIS OF DESIGN ENTAILS

The hypothesis of Intelligent Design (ID) is quite controversial, mostly because of its philosophical and theological overtones, and in the years since *Darwin's Black Box* was published a number of scientists and philosophers have tried to refute its main argument. I have found these rebuttals to be unpersuasive, at best. Quite the opposite, I think that e putative

counterexamples to design are unintentionally instructive. Not only do they fail to make their case for the sufficiency of natural selection, they show clearly the obstacle that irreducible complexity poses to Darwinism. They also show that Darwinists have great trouble recognizing problems with their own theory. I will examine two of those counterexamples in detail a little later in this chapter. Before I do, however, I will first address a few common misconceptions that surround the biochemical design argument.

First of all, it is important to understand that a hypothesis of Intelligent Design has no quarrel with evolution per se – that is, evolution understood simply as descent with modification, but leaving the mechanism open. After all, a designer may have chosen to work that way. Rather than common descent, the focus of ID is on the *mechanism* of evolution – how did all this happen, by natural selection or by purposeful Intelligent Design?

A second point that is often overlooked but should be emphasized is that Intelligent Design can happily coexist with even a large degree of natural selection. Antibiotic and pesticide resistance, antifreeze proteins in fish and plants, and more may indeed be explained by a Darwinian mechanism. The critical claim of ID is not that natural selection doesn't explain *anything*, but that it doesn't explain *everything*.

My book, *Darwin's Black Box*, in which I flesh out the design argument, has been widely discussed in many publications. Although many issues have been raised, I think the general reaction of scientists to the design argument is well and succinctly summarized in the recent book *The Way of the Cell*, published by Oxford University Press and authored by the Colorado State University biochemist Franklin Harold. Citing my book, Harold writes, "We should reject, as a matter of principle, the substitution of intelligent design for the dialogue of chance and necessity (Behe 1996); but we must concede that there are presently no detailed Darwinian accounts of the evolution of any biochemical system, only a variety of wishful speculations" (Harold 2001, 205).

Let me emphasize, in reverse order, Harold's two points. First, as other reviewers of my book have done,[1] Harold acknowledges that Darwinists have no real explanation for the enormous complexity of the cell, only hand-waving speculations, more colloquially known as "just-so stories." I had claimed essentially the same thing six years earlier in *Darwin's Black Box* and encountered fierce resistance – mostly from internet fans of Darwinism who claimed that, why, there were hundreds or thousands of research papers describing the Darwinian evolution of irreducibly complex biochemical systems, and who set up web sites to document them.[2]

As a sufficient response to such claims, I will simply rely on Harold's statement quoted here, as well as the other reviewers who agree that there is a dearth of Darwinian explanations. After all, if prominent scientists who are no fans of Intelligent Design agree that the systems remain unexplained, then that should settle the matter. Let me pause, however, to note that I find



this an astonishing admission for a theory that has dominated biology for so long. That Darwinian theory has borne so little fruit in explaining the molecular basis of life – despite its long reign as the fundamental theory of biology – strongly suggests that it is not the right framework for understanding the origin of the complexity of life.

Harold's second point is that there is some principle that forbids us from investigating Intelligent Design, even though design is an obvious idea that quickly pops into your mind when you see a drawing of the flagellum (Figure 19.1) or other complex biochemical system. What principle is that? He never spells it out, but I think the principle probably boils down to this: design appears to point strongly beyond nature. It has philosophical and theological implications, and that makes many people uncomfortable. Because they think that science should avoid a theory that points so strongly beyond nature, they want to rule out intelligent design from the start.

I completely disagree with that view and find it fainthearted. I think science should follow the evidence wherever it seems to lead. That is the only way to make progress. Furthermore, not only Intelligent Design, but *any* theory that purports to explain how life occurred will have philosophical and theological implications. For example, the Oxford biologist Richard Dawkins has famously said that "Darwin made it possible to be an intellectually-fulfilled atheist" (Dawkins 1986, 6). A little less famously, Kenneth Miller has written that "[God] used evolution as the tool to set us free" (Miller 1999, 253). Stuart Kauffman, a leading complexity theorist, thinks Darwinism cannot explain all of biology: "Darwinism is not enough. . . . [N]atural selection cannot be the sole source of order we see in the world" (Kauffman 1995, viii). But Kauffman thinks that his theory will somehow show that we are "at home in the universe." The point, then, is that all theories of origins carry philosophical and theological implications. There is no way to avoid them in an explanation of life.

Another source of difficulty for some people concerns the question, how could biochemical systems have been designed? A common misconception is that designed systems would have to be created from scratch in a puff of smoke. But that isn't necessarily so. The design process may have been much more subtle. In fact, it may have contravened no natural laws at all. Let's consider just one possibility. Suppose the designer is indeed God, as most people would suspect. Well, then, as Kenneth Miller points out in his book, *Finding Darwin's God*:

> The indeterminate nature of quantum events would allow a clever and subtle God to influence events in ways that are profound, but scientifically undetectable to us. Those events could include the appearance of mutations . . . and even the survival of individual cells and organisms affected by the chance processes of radioactive decay. (Miller 1999, 241)

Miller doesn't think that guidance is necessary in evolution, but if it were (as I believe), then a route would be open for a subtle God to design life without overriding natural law. If quantum events such as radioactive decay are not governed by causal laws, then it breaks no law of nature to influence such events. As a theist like Miller, that seems perfectly possible to me. I would add, however, that such a process would amount to Intelligent Design, not Darwinian evolution. Further, while we might not be able to detect quantum manipulations, we may nevertheless be able to conclude confidently that the final structure was designed.

## MISCONCEPTIONS CONCERNING SUPPOSED WAYS AROUND THE IRREDUCIBILITY OF BIOCHEMICAL SYSTEMS

Consider a hypothetical example where proteins homologous to all of the parts of an irreducibly complex molecular machine first had other individual functions in the cell. Might the irreducible system then have been put together from individual components that originally worked on their own, as some Darwinists have proposed? Unfortunately, this picture greatly oversimplifies the difficulty, as I discussed in *Darwin's Black Box* (Behe 1996, 53). Here analogies to mousetraps break down somewhat, because the parts of a molecular system have to find each other automatically in the cell. They can't be arranged by an intelligent agent, as a mousetrap is. In order to find each other in the cell, interacting parts have to have their surfaces shaped so that they are very closely matched to each other, as pictured in Figure 19.2. Originally, however, the individually acting components would not have had complementary surfaces. So all of the interacting surfaces of

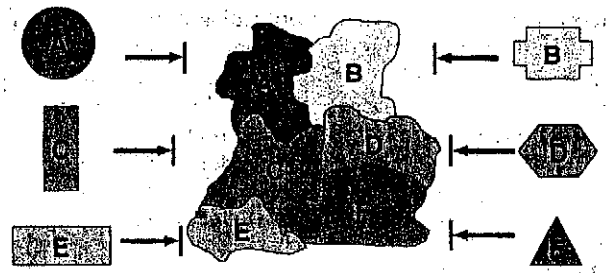


FIGURE 19.2. The parts of an irreducibly complex molecular machine must have surfaces that are closely matched to each other to allow specific binding. This drawing emphasizes that even if individually acting proteins homologous to parts of a complex originally had separate functions, their surfaces would not be complementary to each other. Thus the problem of irreducibility remains even if the separate parts originally had individual functions. (The blocked arrows indicate that the original protein s are not suitable to bind other proteins in the molecular machine.)



all of the components would first have to be adjusted before they could function together. And only then would the new function of the composite system appear. Thus, I emphasize strongly, *the problem of irreducibility remains, even if individual proteins homologous to system components separately and originally had their own functions.*

Another area where one has to be careful is in noticing that some systems that have extra or redundant components may have an irreducibly complex *core.* For example, a car with four spark plugs might get by with three or two, but it certainly can't get by with none. Rat traps often have two springs, to give them extra strength. The trap can still work if one spring is removed, but it can't work if both springs are removed. Thus in trying to imagine the origin of a rat trap by Darwinian means, we still have all the problems we had with a mousetrap. A cellular example of redundancy is the hugely complex eukaryotic cilium, which contains about 250 distinct protein parts (Dutcher 1995). The cilium has multiple copies of a number of components, including multiple microtubules and dynein arms. Yet a working cilium needs at least one copy of each in order to work, as I pictured in my book (Behe 1996, 60). Thus, like the rat trap's, its gradual Darwinian production remains quite difficult to envision. Kenneth Miller has pointed to the redundancy of the cilium as a counterexample to my claim of its irreducibility (Miller 1999, 140–3). But redundancy only delays irreducibility; it does not eliminate it.

Finally, rather than showing how their theory could handle the obstacle, some Darwinists are hoping to get around irreducible complexity by verbal tap dancing. At a debate between proponents and opponents of Intelligent Design sponsored by the American Museum of Natural History in April 2002, Kenneth Miller actually claimed (the transcript is available at the web site of the National Center for Science Education) that a mousetrap isn't irreducibly complex because subsets of a mousetrap, and even each individual part, could still "function" on their own. The holding bar of a mousetrap, Miller observed, could be used as *a toothpick,* so it still has a "function" outside the mousetrap. Any of the parts of the trap could be used as a paperweight, he continued, so they all have "functions." And since any object that has mass can be a paperweight, then any part of anything has a function of its own. *Presto,* there is no such thing as irreducible complexity! Thus the acute problem for gradualism that any child can see in systems like the mousetrap is smoothly explained away.

Of course, the facile explanation rests on a transparent fallacy, a brazen equivocation. Miller uses the word "function" in two different senses. Recall that the definition of irreducible complexity notes that removal of a part "causes the *system* to effectively cease functioning." Without saying so, in his exposition Miller shifts the focus from the separate function of the intact *system* itself to the question of whether we can find a different use (or "function") for some of the *parts.* However, if one removes a part from the mousetrap that I have pictured, it can no longer catch mice. The *system* has

indeed effectively ceased functioning, so the *system* is irreducibly complex, just as I have written. What's more, the functions that Miller glibly assigns to the parts – paperweight, toothpick, key chain, and so forth – have little or nothing to do with the function of the system – catching mice (unlike the mousetrap series proposed by John McDonald, to be discussed later) – so they give us no clue as to how the system's function could arise gradually. Miller has explained precisely nothing.

With the problem of the mousetrap behind him, Miller then moved on to the bacterial flagellum – and again resorted to the same fallacy. If nothing else, one has to admire the breathtaking audacity of verbally trying to turn another severe problem for Darwinism into an advantage. In recent years, it has been shown that the bacterial flagellum is an even more sophisticated system than had been thought. Not only does it act as a rotary propulsion device, it also contains within itself an elegant mechanism used to transport the proteins that make up the outer portion of the machine from the inside of the cell to the outside (Aizawa 1996). Without blinking, Miller asserted that the flagellum is not irreducibly complex because some proteins of the flagellum could be missing and the remainder could still transport proteins, perhaps independently. (Proteins similar – but not identical – to some found in the flagellum occur in the type III secretory system of some bacteria. See Hueck 1998). Again, he was equivocating, switching the focus from the function of the system, acting as a rotary propulsion machine, to the ability of a subset of the system to transport proteins across a membrane. However, taking away the parts of the flagellum certainly destroys the ability of the system to act as a rotary propulsion machine, as I have argued. Thus, contra Miller, the flagellum is indeed irreducibly complex. What's more, the function of transporting proteins has as little directly to do with the function of rotary propulsion as a toothpick has to do with a mousetrap. So discovering the supportive function of transporting proteins tells us precisely nothing about how Darwinian processes might have put together a rotary propulsion machine.

## THE BLOOD CLOTTING CASCADE

Having dealt with some common misconceptions about intelligent design, in the next two sections I will examine two systems that were proposed as serious counterexamples to my claim of irreducible complexity. I will show not only that they fail, but also how they highlight the seriousness of the obstacle of irreducible complexity.

In *Darwin's Black Box*, I argued that the blood clotting cascade is an example of an irreducibly complex system (Behe 1996, 74–97). At first glance, clotting seems to be a simple process. A small cut or scrape will bleed for a while and then slow down and stop as the visible blood congeals. However, stud[illegible]over the past fifty years have shown that the visible simplicity is



undergirded by a system of remarkable complexity (Halkier 1992). In all, there are over a score of separate protein parts involved in the vertebrate clotting system. The concerted action of the components results in the formation of a weblike structure at the site of the cut, which traps red blood cells and stops the bleeding. Most of the components of the clotting cascade are involved not in the structure of the clot itself, but in the control of the timing and placement of the clot. After all, it would not do to have clots forming at inappropriate times and places. A clot that formed in the wrong place, such as in the heart or brain, could lead to a heart attack or stroke. Yet a clot that formed even in the right place, but too slowly, would do little good.

The insoluble weblike fibers of the clot material itself are formed of a protein called fibrin. However, an insoluble web would gum up blood flow before a cut or scrape happened, so fibrin exists in the bloodstream initially in a soluble, inactive form called fibrinogen. When the closed circulatory system is breached, fibrinogen is activated by having a piece cut off from one end of two of the three proteins that comprise it. This exposes sticky sites on the protein, which allows them to aggregate. Because of the shape of the fibrin, the molecules aggregate into long fibers that form the meshwork of the clot. Eventually, when healing is completed, the clot is removed by an enzyme called plasmin.

The enzyme that converts fibrinogen to fibrin is called thrombin. Yet the action of thrombin itself has to be carefully regulated. If it were not, then thrombin would quickly convert fibrinogen to fribrin, causing massive blood clots and rapid death. It turns out that thrombin exists in an inactive form called prothrombin, which has to be activated by another component called Stuart factor. But by the same reasoning, the activity of Stuart factor has to be controlled, too, and it is activated by yet another component. Ultimately, the component that usually begins the cascade is tissue factor, which occurs on cells that normally do not come in contact with the circulatory system. However, when a cut occurs, blood is exposed to tissue factor, which initiates the clotting cascade.

Thus in the clotting cascade, one component acts on another, which acts on the next, and so forth. I argued that the cascade is irreducibly complex because, if a component is removed, the pathway is either immediately turned on or permanently turned off. It would not do, I wrote, to postulate that the pathway started from one end, fibrinogen, and then added components, since fibrinogen itself does no good. Nor is it plausible even to start with something like fibrinogen and a nonspecific enzyme that might cleave it, since the clotting would not be regulated and would be much more likely to do harm than good.

So said I. But Russell Doolittle – an eminent protein biochemist, a professor of biochemistry at the University of California–San Diego, a member of the National Academy of Sciences, and a lifelong student [illegible]e blood

clotting system – disagreed. As part of a symposium discussing my book and Richard Dawkins' *Climbing Mount Improbable* in the *Boston Review*, which is published by the Massachusetts Institute of Technology, Doolittle wrote an essay discussing the phenomenon of gene duplication – the process by which a cell may be provided with an extra copy of a functioning gene. He then conjectured that the components of the blood clotting pathway, many of which have structures that are similar to each other, arose by gene duplication and gradual divergence. This is the common view among Darwinists. Professor Doolittle went on to describe a then-recent experiment that, he thought, showed that the cascade is not irreducible after all. Professor Doolittle cited a paper by Bugge and colleagues (1996a) entitled "Loss of Fibrinogen Rescues Mice from the Pleiotropic Effects of Plasminogen Deficiency." Of that paper, he wrote:

> Recently the gene for plaminogen [sic] was knocked out of mice, and, predictably, those mice had thrombotic complications because fibrin clots could not be cleared away. Not long after that, the same workers knocked out the gene for fibrinogen in another line of mice. Again, predictably, these mice were ailing, although in this case hemorrhage was the problem. And what do you think happened when these two lines of mice were crossed? For all practical purposes, the mice lacking both genes were normal! Contrary to claims about irreducible complexity, the entire ensemble of proteins is not needed. Music and harmony can arise from a smaller orchestra. (Doolittle 1997)

(Again, fibrinogen is the precursor of the clot material itself. Plasminogen is the precursor of plasmin, which removes clots once their purpose is accomplished.) So if one knocks out either one of those genes of the clotting pathway, trouble results; but, Doolittle asserted, if one knocks out both, then the system is apparently functional again. That would be a very interesting result, but it turns out to be incorrect. Doolittle misread the paper.

The abstract of the paper states that "[m]ice deficient in plasminogen and fibrinogen are phenotypically indistinguishable from fibrinogen-deficient mice." In other words, the double mutants have all the problems that the mice lacking just fibrinogen have. Those problems include inability to clot, hemorrhaging, and death of females during pregnancy. Plasminogen deficiency leads to a different suite of symptoms – thrombosis, ulcers, and high mortality. Mice missing both genes were "rescued" from the ill effects of plasminogen deficiency only to suffer the problems associated with fibrinogen deficiency.[3] The reason for this is easy to see. Plasminogen is needed to remove clots that, left in place, interfere with normal functions. However, if the gene for fibrinogen is also knocked out, then clots can't form in the first place, and their removal is not an issue. Yet if clots can't form, then there is no functioning clotting system, and the mice suffer the predictable consequences.



TABLE 19.1. *Effects of knocking out genes for blood clotting components*

| Missing Protein | Symptoms | Reference |
|---|---|---|
| Plasminogen | Thrombosis, high mortality | Bugge et al. 1995 |
| Fibrinogen | Hemorrhage, death in pregnancy | Suh et al. 1995 |
| Plasminogen/fibrinogen | Hemorrhage, death in pregnancy | Bugge et al. 1996a |
| Prothrombin | Hemorrhage, death in pregnancy | Sun et al. 1998 |
| Tissue factor | Hemorrhage, death in pregnancy | Bugge et al. 1996b |

Clearly, the double-knockout mice are not "normal." They are not promising evolutionary intermediates.

The same group that produced the mice missing plasminogen and fibrinogen has also produced mice individually missing other components of the clotting cascade – prothrombin and tissue factor. In each case, the mice are severely compromised, which is *exactly* what one would expect if the cascade is irreducibly complex (Table 19.1).

What lessons can we draw from this incident? The point is certainly not that Russell Doolittle misread a paper, which anyone might do. (Scientists, as a rule, are not known for their ability to write clearly, and Bugge and colleagues were no exception.) Rather, the main lesson is that irreducible complexity seems to be a much more severe problem than Darwinists recognize, since the experiment Doolittle himself chose to demonstrate that "music and harmony can arise from a smaller orchestra" showed exactly the opposite. A second lesson is that gene duplication is not the panacea that it is often made out to be. Professor Doolittle knows as much about the structures of the clotting proteins and their genes as anyone on Earth, and he is convinced that many of them arose by gene duplication and exon shuffling. Yet that knowledge did not prevent him from proposing utterly nonviable mutants as possible examples of evolutionary intermediates. A third lesson is that, as I had claimed in *Darwin's Black Box*, there are no papers in the scientific literature detailing how the clotting pathway could have arisen by Darwinian means. If there were, Doolittle would simply have cited them.

Another significant lesson that we can draw is that, while the majority of academic biologists and philosophers place their confidence in Darwinism, that confidence rests on no firmer grounds than Professor Doolittle's. As an illustration, consider the words of the philosopher Michael Ruse:

> For example, Behe is a real scientist, but this case for the impossibility of a small-step natural origin of biological complexity has been trampled upon contemptuously by the scientists working in the field. They think his grasp of the pertinent science is weak and his knowledge of the literature curiously (although conveniently) outdated.

For example, far from the evolution of clotting being a mystery, the past three decades of work by Russell Doolittle and others has thrown significant light on the ways in which clotting came into being. More than this, it can be shown that the clotting mechanism does not have to be a one-step phenomenon with everything already in place and functioning. One step in the cascade involves fibrinogen, required for clotting, and another, plaminogen [*sic*], required for clearing clots away. (Ruse 1998)

And Ruse goes on to quote Doolittle's passage from the *Boston Review* that I quoted earlier. Now, Ruse is a prominent Darwinist and has written many books on various aspects of Darwiniana. Yet, as his approving quotation of Doolittle's mistaken reasoning shows (complete with his copying of Doolittle's typo-misspelling of "plaminogen"), Ruse has no independent knowledge of how natural selection could have put together complex biochemical systems. As far as the scientific dispute is concerned, Ruse has nothing to add.

Another such example is seen in a recent essay in *The Scientist*, "Not-So-Intelligent Design," by Neil S. Greenspan, a professor of pathology at Case Western Reserve University, who writes (Greenspan 2002), "The Design advocates also ignore the accumulating examples of the reducibility of biological systems. As Russell Doolittle has noted in commenting on the writings of one ID advocate . . ." Greenspan goes on to cite approvingly Doolittle's argument in the *Boston Review*. He concludes, with unwitting irony, that "[t]hese results cast doubt on the claim by proponents of ID that they know which systems exhibit irreducible complexity and which do not." But since the results are precisely the opposite of what Greenspan supposed, the shoe is now on the other foot. This incident casts grave doubt on the claim by Darwinists – both biologists and philosophers – that they know that complex cellular systems are explainable in Darwinian terms. It demonstrates that Darwinists either cannot or will not recognize difficulties for their theory.

## THE MOUSETRAP

The second counterargument to irreducibility I will discuss here concerns not a biological example but a conceptual one. In *Darwin's Black Box*, I pointed to a common mechanical mousetrap as an example of irreducible complexity. Almost immediately after the book's publication, some Darwinists began proposing ways in which the mousetrap could be built step by step. One proposal that has gotten wide attention, and that has been endorsed by some prominent scientists, was put forward by John McDonald, a professor of biology at the University of Delaware, and can be seen on his web site.[4] His series of traps is shown in Figure 19.3. McDonald's main point was that the trap that I pictured in my book consisted of five parts, yet he could b a trap with fewer parts.



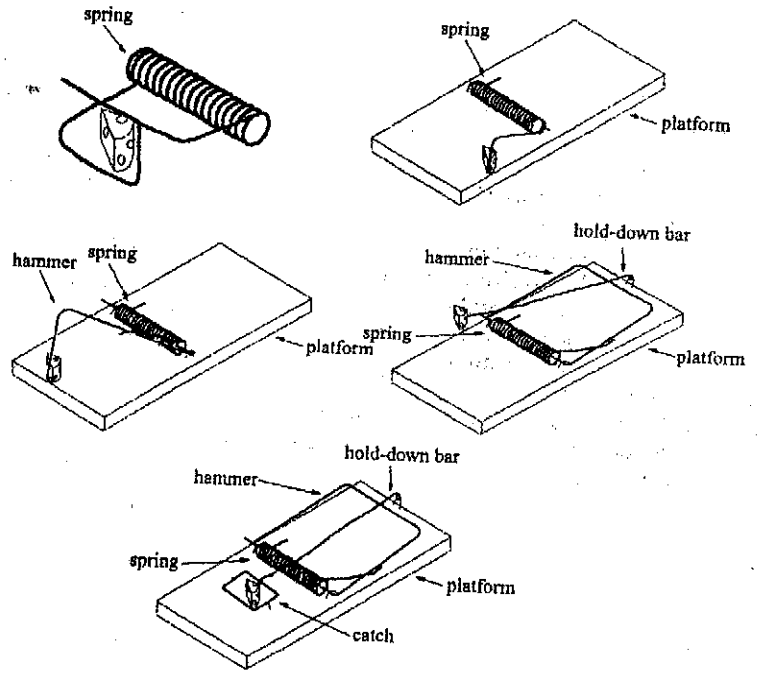


FIGURE 19.3. A series of mousetraps with an increasing number of parts, as proposed by John McDonald <http://udel.edu/~mcdonald/oldmousetrap.html> and reproduced here with his permission. Yet intelligence is still required to construct one trap from another, as described in the text.

I agree. In fact, I said exactly the same thing in my book. I wrote:

We need to distinguish between a *physical* precursor and a *conceptual* precursor. The trap described above is not the only system that can immobilize a mouse. On other occasions my family has used a glue trap. In theory at least, one can use a box propped open with a stick that could be tripped. Or one can simply shoot the mouse with a BB gun. However, these are not physical precursors to the standard mousetrap since they cannot be transformed, step-by-Darwinian-step, into a trap with a base, hammer, spring, catch, and holding bar. (Behe 1996, 43)

Thus the point is not that mousetraps can be built in different ways, with different numbers of pieces. (My children have a game at home called "Mousetrap," which has many, many pieces and looks altogether different from the common mechanical one.) Of course they can. The only questi whether a particular trap can he built by "numerous, successive, slight n ications"

to a simple starting point – without the intervention of intelligence – as Darwin insisted that his theory required.

The McDonald traps cannot. Shown at the top of Figure 19.3 are his one-piece trap and his two-piece trap. The structure of the second trap, however, is not a single, small, random step away from the first. First notice that the one-piece trap is not a simple spring – it is shaped in a very special way. In fact, the shape was deliberately chosen by an intelligent agent, John McDonald, to act as a trap. Well, one has to start somewhere. But if the mousetrap series is to have any relevance at all to Darwinian evolution, then intelligence can't be involved at any further point.

Yet intelligence saturates the whole series. Consider what would be necessary to convert the one-piece trap to the "two-piece" trap. One can't just place the first trap on a simple piece of wood and have it work as the second trap does. Rather, as shown in Figure 19.3, the two protruding ends of the spring first have to be reoriented. What's more, two staples (barely visible in Figure 19.3) are added to hold the spring onto the platform so that it can be under tension in the two-piece trap. So we have gone not from a one-piece to a two-piece trap, but from a one-piece to a four-piece trap. Notice also that the placement of the staples in relation to the edge of the platform is critical. If the staples were moved a quarter-inch from where they are, the trap wouldn't work. Finally, consider that, in order to have a serious analogy to the robotic processes of the cell, we can't have an intelligent human setting the mousetrap – the first trap would have to be set by some unconscious charging mechanism. So, when the pieces are rearranged, the charging mechanism too would have to change for the second trap.

It's easy for us intelligent agents to overlook our role in directing the construction of a system, but nature cannot overlook any step at all, so the McDonald mousetrap series completely fails as an analogy to Darwinian evolution. In fact, the second trap is best viewed not as some Darwinian descendant of the first but as a completely different trap, designed by an intelligent agent, perhaps using a refashioned part or two from the first trap.

Each of the subsequent steps in the series suffers from analogous problems, which I have discussed elsewhere.[5]

In his endorsement of the McDonald mousetrap series, Kenneth Miller wrote: "If simpler versions of this mechanical device [the mousetrap] can be shown to work, then simpler versions of biochemical machines could work as well . . . and this means that complex biochemical machines could indeed have had functional precursors."[6] But that is exactly what it doesn't show – if by "precursor" Miller means "Darwinian precursor." On the contrary, McDonald's mousetrap series shows that even if one does find a simpler system to perform some function, that gives one no reason to think that a more complex system performing the same function could be produced by a Darwinian process starting with the simpler system. Rather, the difficulty in doing so for a simple mousetrap gives us compelling reason to think it cannot be done for complex molecular machines.



## FUTURE PROSPECTS OF THE INTELLIGENT DESIGN HYPOTHESIS

The misconceived arguments by Darwinists that I have recounted here offer strong encouragement to me that the hypothesis of Intelligent Design is on the right track. After all, if well-informed opponents of an idea attack it by citing data that, when considered objectively, actually demonstrate its force, then one is entitled to be confident that the idea is worth investigating.

Yet it is not primarily the inadequacy of Darwinist responses that bodes well for the design hypothesis. Rather, the strength of design derives mainly from the work-a-day progress of science. In order to appreciate this fact, it is important to realize that the idea of Intelligent Design arose not from the work of any individual but from the collective work of biology, particularly in the last fifty years. Fifty years ago, the cell seemed much simpler, and in our innocence it was easier then to think that Darwinian processes might have accounted for it. But as biology progressed and the imagined simplicity vanished, the idea of design became more and more compelling. That trend is continuing inexorably. The cell is not getting any simpler; it is getting much more complex. I will conclude this chapter by citing just one example, from the relatively new area of proteomics.

With the successful sequencing of the entire genomes of dozens of microorganisms and one vertebrate (us), the impetus has turned toward analyzing the cellular interactions of the proteins that the genomes code for, taken as a whole. Remarkable progress has already been made. Early in 2002, an exhaustive study of the proteins comprising the yeast proteome was reported. Among other questions, the investigators asked what proportion of yeast proteins work as groups. They discovered that nearly fifty percent of proteins work as complexes of a half-dozen or more, and many as complexes of ten or more (Gavin et al. 2002).

This is not at all what Darwinists had expected. As Bruce Alberts wrote earlier in the article "The Cell as a Collection of Protein Machines":

> We have always underestimated cells. Undoubtedly we still do today. But at least we are no longer as naive as we were when I was a graduate student in the 1960s. Then most of us viewed cells as containing a giant set of second-order reactions. . . .
>
> But, as it turns out, we can walk and we can talk because the chemistry that makes life possible is much more elaborate and sophisticated than anything we students had ever considered. Proteins make up most of the dry mass of a cell. But instead of a cell dominated by randomly colliding individual protein molecules, we now know that nearly every major process in a cell is carried out by assemblies of 10 or more protein molecules. And, as it carries out its biological functions, each of these protein assemblies interacts with several other large complexes of proteins.

Indeed, the entire cell can be viewed as a factory that contains an elaborate network of interlocking assembly lines, each of which is composed of a set of large protein machines. (Alberts 1998)

The important point here for a theory of Intelligent Design is that molecular machines are not confined to the few examples that I discussed in *Darwin's Black Box*. Rather, most proteins are found as components of complicated molecular machines. Thus design might extend to a large fraction of the features of the cell, and perhaps beyond that into higher levels of biology.

Progress in twentieth-century science has led us to the design hypothesis. I expect progress in the twenty-first century to confirm and extend it.

## Notes

1. For example, the microbiologist James Shapiro of the University of Chicago declared in *National Review* that "[t]here are no detailed Darwinian accounts for the evolution of any fundamental biochemical or cellular system, only a variety of wishful speculations" (Shapiro 1996, 65). In *Nature*, the University of Chicago evolutionary biologist Jerry Coyne stated, "There is no doubt that the pathways described by Behe are dauntingly complex, and their evolution will be hard to unravel.... [W]e may forever be unable to envisage the first proto-pathways" (Coyne 1996, 227). In a particularly scathing review in *Trends in Ecology and Evolution*, Tom Cavalier-Smith, an evolutionary biologist at the University of British Columbia, nonetheless wrote, "For none of the cases mentioned by Behe is there yet a comprehensive and detailed explanation of the probable steps in the evolution of the observed complexity. The problems have indeed been sorely neglected – though Behe repeatedly exaggerates this neglect with such hyperboles as 'an eerie and complete silence' " (Cavalier-Smith 1997, 162). The Evolutionary biologist Andrew Pomiankowski, writing in *New Scientist*, agreed: "Pick up any biochemistry textbook, and you will find perhaps two or three references to evolution. Turn to one of these and you will be lucky to find anything better than 'evolution selects the fittest molecules for their biological function' " (Pomiankowski 1996, 44). In *American Scientist*, the Yale molecular biologist Robert Dorit averred, "In a narrow sense, Behe is correct when he argues that we do not yet fully understand the evolution of the flagellar motor or the blood clotting cascade" (Dorit 1997, 474).
2. A good example is found on the "World of Richard Dawkins" web site, maintained by a Dawkins fan named John Catalano at <www.world-of-dawkins.com/Catalano/box/published.htm>. It is to this site that the Oxford University physical chemist Peter Atkins was referring when he wrote in a review of *Darwin's Black Box* for the "Infidels" web site: "Dr. Behe claims that science is largely silent on the details of molecular evolution, the emergence of complex biochemical pathways and processes that underlie the more traditional manifestations of evolution at the level of organisms. Tosh! There are hundreds, possibly thousands, of scientific papers that deal with this very subject. For an entry into this important and flourishing field, and an idea of the intense scientific effort that it repr s (see the first link above) [*sic*]" (Atkins 1998).



3. Bugge and colleagues (1996a) were interested in the question of whether plasminogen had any role in metabolism other than its role in clotting, as had been postulated. The fact that the direct effects of plasminogen deficiency were ameliorated by fibrinogen deficiency showed that plasminogen probably had no other role.
4. <http://udel.edu/~mcdonald/oldmousetrap.html>. Professor McDonald has recently designed a new series of traps that can be seen at <http://udel.edu/~mcdonald/mousetrap.html>. I have examined them and have concluded that they involve his directing intelligence to the same degree.
5. M. J. Behe, "A Mousetrap Defended: Response to Critics." <www.crsc.org>
6. <http://biocrs.biomed.brown.edu/Darwin/DI/Mousetrap.html>

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# Exhibit 10

*Protein Science* journal article

# Simulating evolution by gene duplication of protein features that require multiple amino acid residues

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**Abstract**

Gene duplication is thought to be a major source of evolutionary innovation because it allows one copy of a gene to mutate and explore genetic space while the other copy continues to fulfill the original function. Models of the process often implicitly assume that a single mutation to the duplicated gene can confer a new selectable property. Yet some protein features, such as disulfide bonds or ligand binding sites, require the participation of two or more amino acid residues, which could require several mutations. Here we model the evolution of such protein features by what we consider to be the conceptually simplest route—point mutation in duplicated genes. We show that for very large population sizes $N$, where at steady state in the absence of selection the population would be expected to contain one or more duplicated alleles coding for the feature, the time to fixation in the population hovers near the inverse of the point mutation rate, and varies sluggishly with the $\lambda^{th}$ root of $1/N$, where $\lambda$ is the number of nucleotide positions that must be mutated to produce the feature. At smaller population sizes, the time to fixation varies linearly with $1/N$ and exceeds the inverse of the point mutation rate. We conclude that, in general, to be fixed in $10^8$ generations, the production of novel protein features that require the participation of two or more amino acid residues simply by multiple point mutations in duplicated genes would entail population sizes of no less than $10^9$.

**Keywords:** gene duplication; point mutation; multiresidue feature; disulfide bonds; ligand binding sites

Although many scientists assume that Darwinian processes account for the evolution of complex biochemical systems, we are skeptical. Thus, rather than simply assuming the general efficacy of random mutation and selection, we want to examine, to the extent possible, which changes are reasonable to expect from a Darwinian process and which are not. We think the most tractable place to begin is with questions of protein structure. Our approach is to examine pathways that are currently considered to be likely routes of evolutionary development and see what types of changes Darwinian processes may be expected to promote along a particular pathway.

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*Abbreviation*: MR, multiresidue

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A major route of evolutionary innovation is thought to pass through gene duplication (Ohno 1970; Lynch and Conery 2000; Wagner 2001; Chothia et al. 2003). Because one copy of the gene can continue to fulfill the original function, in this view a duplicate, redundant copy of a gene is substantially free from purifying selection, allowing it to freely accumulate mutations. Although the great majority of nonneutral mutations to duplicated genes are expected to result in a null allele (Walsh 1995; Lynch and Walsh 1998), that is, a gene that no longer codes for a functional protein, occasionally one might confer a novel function on the incipient paralog. If this occurs, then the duplicated gene can be refined by mutation and positive selection, independent of the parent gene.

In most models of the development of evolutionary novelty by gene duplication, it is implicitly assumed that a single, albeit rare, mutation to the duplicated gene can confer a new selectable property (Ohta 1987, 1988a,b; Walsh 1995). However, we are particularly interested in the ques-

tion of how novel protein structural features may develop throughout evolution; not all structural features of a protein may be attainable by single mutations. In particular, some protein features require the participation of multiple amino acid residues. Perhaps the simplest example of this is the disulfide bond. In order to produce a novel disulfide bond, a duplicated gene coding for a protein lacking unmatched cysteines would require at least two mutations in separate codons, and perhaps as many as six mutations, depending on the starting codons. We call protein characteristics such as disulfide bonds which require the participation of two or more amino acid residues "multiresidue" (MR) features.

A more general example of an MR feature is that of a protein binding site. A ligand bound to a protein interacts with multiple amino acid residues (Janin and Chothia 1990; Cunningham and Wells 1993; Braden and Poljak 1995; Lo et al. 1999; Chakrabarti and Janin 2002). In general, therefore, in order to produce a binding site for a new ligand in a protein originally lacking the ability to bind it, multiple mutational events would be necessary. Li (1997) drew attention to this fact in his textbook *Molecular Evolution*. Prefacing a discussion of the evolutionary development of the 2,3-diphosphoglycerate binding site of hemoglobin, he wrote, "acquiring a new function may require many mutational steps, and a point that needs emphasis is that the early steps might have been selectively neutral because the new function might not be manifested until a certain number of steps had already occurred" (Li 1997).

In this paper, we report the results of the stochastic simulation of the time to fixation of new MR features by what we consider to be the conceptually simplest route: point mutation in the absence of recombination in a duplicated gene that is free of purifying selection. It can be seen that, for very large populations, the expected time to fixation resides near the inverse of the mutation rate per nucleotide and decreases only slowly with the $\lambda^{th}$ root of increasing population size, where $\lambda$ is the number of nucleotide positions that must be mutated to produce the feature. For smaller populations, the time varies linearly with $1/N$.

## Results

### *The model*

The model presented here assumes that newly duplicated genes encode a full-length protein with the signals necessary for its proper expression. It is further assumed that all duplicate genes are selectively neutral. (This postulate is examined in the Discussion.) Any given organism in the population may be thought to have anywhere from zero to multiple extra copies of the gene; that is, duplicate copy number is considered to have no selective effect. However, the model presupposes that there are a total of $N$ duplicate copies of the gene, equal to the number of organisms in the population. The model assumes that either copy of a newly duplicated gene can be the one to undergo mutation and that either copy can retain the original function. That is, the original gene is not necessarily the one to retain the original function. Because the model does not include recombination, all copies of the gene accumulate point mutations independently of each other. The basic "task" that the model asks a duplicate gene to perform is to accumulate $\lambda$ mutations at the correct nucleotide positions to code for a new selectable feature before suffering a null mutation. Because the model presented here does not include recombination, the results can be considered to be most applicable to a haploid, asexual population. However, as will be discussed, implications can also be made for the evolution of diploid, sexual species.

The process we envision for the production of a multiresidue (MR) feature is illustrated in Figure 1, where a duplicate gene coding for a protein is represented as an array of squares that stand for nucleotide positions. A gene coding for a duplicate, redundant protein would contain many nucleotides. The majority of nonneutral point muta-

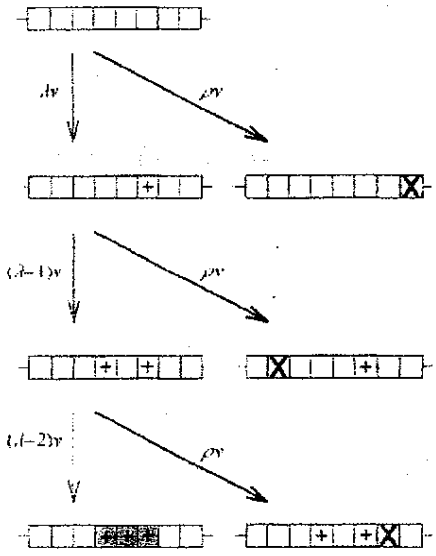


**Figure 1.** A freshly duplicated gene must accrue several compatible mutations without suffering a null mutation in order to code for the multiresidue (MR) feature. Each box in an array represents a nucleotide position in the duplicated gene. The three boxes outlined in blue are the positions that must be changed in order to produce the new MR feature. (Although they are contiguous in the drawing, they do not necessarily represent contiguous positions in the gene.) A "+" labels a compatible mutation. A red "X" labels a null mutation. The green-shaded box represents the gene coding for the MR feature, where the several necessary changes have all been acquired. The forward mutation rate is $v$ times the number of incompatible loci $\lambda$ remaining to be changed. The null mutation rate is $\mu v$.

tions to the gene will yield a null allele (again, by which we mean a gene coding for a nonfunctional protein) because most mutations that alter the amino acid sequence of a protein effectively eliminate function (Reidhaar-Olson and Sauer 1988, 1990; Bowie and Sauer 1989; Lim and Sauer 1989; Bowie et al. 1990; Rennell et al. 1991; Axe et al. 1996; Huang et al. 1996; Sauer et al. 1996; Suckow et al. 1996). However, if several point mutations (indicated by a "+" in the figure) accumulate at specific nucleotide positions (indicated by the three squares outlined in blue in the figure) in the gene coding for the protein before a null mutation occurs elsewhere in the gene (indicated by a red "X"), then several amino acid residues will have been altered and the new selectable MR feature will have been successfully built in the protein (indicated by the green-shaded area). By hypothesis, the gene is not selectable for the new feature when an intermediate number of mutations has occurred, but only when all sites are in the correct state.

In our computer model of the process described above, the nucleotide positions that must be changed from the sequence of the parent gene to be compatible with the developing MR feature (we call states of nucleotide positions "compatible" if they are consistent with what is necessary to code for the MR feature, and "incompatible" if they are not) are explicitly represented as elements of an array (see Materials and Methods for details). These correspond to the squares outlined in blue in Figure 1. (Although the positions are next to each other in the figure, they are not necessarily contiguous in the gene.) These may be considered to be nucleotide positions in the same codon, separate codons, or a combination. The pertinent feature of the model is that multiple changes are required in the gene before the new, selectable feature appears. Changes in these nucleotide positions are assumed to be individually disruptive of the original function of the protein but are assumed either to enhance the original function or to confer a new function once all are in the compatible state. Thus, the mutations would be strongly selected against in an unduplicated gene, because its function would be disrupted and no duplicate would be available to back up the function.

The other nucleotide positions in the gene, corresponding to the black squares in Figure 1, which if they were changed would yield a null allele, are represented only implicitly in our computer model by the constant $\rho$, which is the ratio of the number of mutations of the original duplicated gene that would produce a null allele to the number of mutations of the original duplicated gene that would yield a compatible residue. (Definitions of terms are given in Table 1.) As an example, consider a gene of a thousand nucleotides. If a total of 2400 point mutations of those positions would yield a null allele, whereas three positions must be changed to build a new MR feature such as a disulfide bond, then $\rho$ would be 2400/3, or 800. (Any possible mutations which are neutral are ignored.) In each generation of the simulation, each of the three positions that must be changed to yield the MR feature is sequentially given a chance to mutate with a probability governed by the mutation rate. However, although a mutation may occur in a position needed for an MR feature, it would nonetheless be unproductive if a null mutation had first occurred at a separate position. To simulate this possibility in our model, when an explicitly represented position does mutate, then we take a further probabilistic step to decide if a null mutation has in the meantime occurred elsewhere in the gene, in positions not explicitly represented. In the earlier example, if one of the three positions mutates, then a further step decides with probability $\rho / (1 + \rho)$ (which in the example would be 800/801) that one or more null mutations have already occurred somewhere in the gene, and the gene is considered to be irrecoverably lost. (The likelihood of a null mutation reverting and the gene then successfully developing an MR feature before other null mutations occur is much lower than if the first $\lambda$ mutations to the duplicate gene yield compatible residues; thus, we ignore that possibility.) With probability $1 / (1 + \rho)$ (in the example this would be 1/801), the gene is considered to be free of null mutations and continues in the simulation.

The starting point of the simulation (see Materials and Methods for a more complete description) is a population of organisms that already contains $N$ exact duplicates of the parent gene, which then begin to undergo mutation. For simplicity, each position in an array, representing sites which must be changed to yield an MR feature, can be in either of just two states—the original incompatible state or the mutated, compatible state. Mutations can change a site

**Table 1.** *Definitions of terms*

| | |
|---|---|
| $N$ | Number of organisms/duplicate genes in the population |
| $\lambda$ | Number of initially incompatible nucleotide loci in a duplicate gene that must be changed to form the selectable, multiresidue feature |
| $\nu$ | Point mutation rate per nucleotide per generation |
| $\rho$ | Ratio of the number of possible mutations of the original duplicated gene that would produce a null allele to the number of possible mutations of the original duplicated gene that would yield a compatible residue. Neutral mutations, such as those that produce synonymous codons, are disregarded. |
| $\phi$ | Fraction of a particular nucleotide position that is in the incompatible state. $(1 - \phi)$ is the fraction in the compatible state. |
| $t$ | Time, in generations |
| $T_f$ | Time in generations to the first occurrence of a particular multiresidue, selectable features |
| $T_{fx}$ | Time in generations to fixation in the population of a particular multiresidue, selectable feature |
| $s$ | Selection coefficient |

either forward from incompatible to compatible or backward from compatible to incompatible. (Unlike for null mutations, reversions of compatible mutations back to incompatible ones must be explicitly considered because the probability of reversion in this case is significant.) These transitions occur with equal intrinsic probabilities.

Starting from a uniform population in which all sites that must be changed are in a state incompatible with the MR feature, then there are three processes in our model which affect the rate of approach of the population to steady state, which in turn affect the time required to generate the new MR feature:

1. Sites in the incompatible state can mutate to the compatible state before any null mutation has occurred. This takes place at a rate equal to the mutation rate per site times the fraction of sites that are in the incompatible state (since only that fraction can mutate directly to the compatible state) times the probability that no null mutation has already occurred. That is, at a rate equal to

$$\nu\phi\left(\frac{1}{1+\rho}\right),$$

where $\nu$ is the mutation rate per site per generation, $\phi$ is the fraction of nucleotide sites in the population that are in the incompatible state, $\rho$ (as mentioned above) is the ratio of possible null to compatible mutations over the entire protein, and $1/(1+\rho)$ is the probability that a compatible mutation occurs before a null mutation. (Definitions of terms are given in Table 1.)

2. A site in the compatible state can mutate back to the incompatible state before a null mutation occurs. This takes place at a rate equal to

$$\nu(1-\phi)\left(\frac{1}{1+\rho}\right).$$

3. A mutation can occur in any one of the $\lambda$ sites, but a stochastic check at this point decides with probability $\rho/(1+\rho)$ that one or more detrimental mutations have already occurred somewhere else in the protein, rendering it nonfunctional. The gene is then considered to be null, and it no longer counts in the model. However, the model allows for the occurrence of new gene duplication events, which recent estimates have shown to happen at a rate comparable to that of point mutation (Lynch and Conery 2000). Because the rates of point mutations and gene duplication are similar, in the model a gene that is determined to be null is replaced by a new gene duplication event, with a new copy of the original gene (which is presumed to be still under selection) with all sites in the original, incompatible state. In the computer model, this process effectively results in all $\lambda$ sites of a null gene being reset to the original, incompatible state from whatever state they were in. This will happen at rate

$$\nu\lambda(1-\phi)\left(\frac{\rho}{1+\rho}\right).$$

The number of nucleotide positions $\lambda$ appears in this expression because the more compatible positions that were contained in a discarded null gene, the more that are replaced with incompatible ones in a new gene duplication event. The protocol of checking for null mutations in the model only when a mutation first occurs in one of the $\lambda$ array sites has the intended effect of ensuring that gene duplication occurs in the population at a rate that is comparable to the rate of point mutation.

The overall net rate of change of the fraction $\phi$ of sites from the incompatible state will be a sum of these three processes:

$$\frac{d\phi}{dt}=\frac{-\nu\phi}{1+\rho}+\frac{\nu(1-\phi)}{1+\rho}+\frac{\nu\rho\lambda(1-\phi)}{1+\rho} \qquad (1)$$

The first term of the right-hand side of the equation is negative because it is a process in which incompatible sites are removed. The second and third terms are positive because they describe processes where incompatible sites are gained.

Integration yields:

$$(1-\phi)=\frac{1-\exp\left(-\nu t\left(\frac{2+\rho\lambda}{1+\rho}\right)\right)}{2+\rho\lambda} \qquad (2)$$

The numerator of the right-hand term is the degree of saturation of the population with compatible mutations—the degree to which it has approached steady state. The value of $(1-\phi)$ is the population-wide fraction of nucleotide positions that are in a state compatible with the MR feature.

Because of computing limitations, the values of 0.01–0.0001 used for the mutation rate $\nu$ in the simulations presented following are much higher than the biologically realistic value of about $10^{-8}$ (Drake et al. 1998), and the values of 1–100 used here for $\rho$ are lower than the value of a thousand or greater expected for biologically realistic situations (Walsh 1995). However, the fact that Figure 2 shows that the fraction $(1-\phi)$ of compatible mutants in our simulations follows equation 2 very closely over a wide range of values for $\lambda$ and $\rho$ in populations that reproduce either deterministically or stochastically makes us more confident

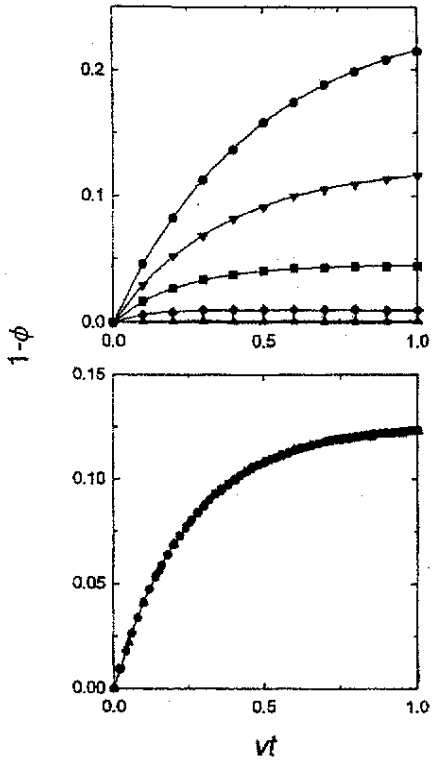


**Figure 2.** Fraction $(1-\phi)$ of a nucleotide position in a compatible state versus time (generations) normalized for the mutation rate ($\nu t$). In all cases, the curves are determined from equation 2. (*Top*) $N = 10{,}000$, $\nu = 0.001$, deterministic reproduction. Circles: $\rho = 1$, $\lambda = 2$; inverted triangles: $\rho = 2$, $\lambda = 3$; squares: $\rho = 4$, $\lambda = 5$; diamonds: $\rho = 10$, $\lambda = 10$; triangles: $\rho = 100$, $\lambda = 10$. Each point is the average of 100 repetitions. (*Bottom*) $N = 100$, $\nu = 0.001$, $\rho = 1$, $\lambda = 6$. Circles are for deterministic reproduction; each point is the average of 100 repetitions. Triangles are for stochastic reproduction; each point is the average of 1024 repetitions.

when we extrapolate the model to biologically realistic values of $\nu$ and $\rho$.

In the following paragraphs, we develop from simple considerations an equation which gives the same quantitative behavior as the numerical model. In Appendix 1, we derive the same form of equation more rigorously by considering coupled equations representing different segments of the population.

What is the probability that a duplicated gene will give rise to a particular MR feature? Consider a gene with $\lambda$ sites all originally in the incompatible state. As discussed previously, the probability of one of those sites mutating to a compatible state before the occurrence of a null mutation elsewhere in the gene is

$$\frac{1}{(1+\rho)}.$$

Because any one of the $\lambda$ sites can mutate first, we can write this as

$$\frac{\lambda}{\lambda}\frac{1}{(1+\rho)}.$$

To mutate another residue to a compatible state, we must choose among the remaining $(\lambda - 1)$ possibilities. Thus, the probability for the second position is

$$\frac{(\lambda-1)}{\lambda}\frac{1}{(1+\rho)}.$$

The multiplied probability of all $\lambda$ sites mutating to compatible states before a null mutation occurs and before a back mutation occurs is thus

$$\frac{\lambda!}{\lambda^{\lambda}}\frac{1}{(1+\rho)^{\lambda}}.$$

(If a back mutation occurs at any point, the likelihood of successfully developing an MR feature is much lower than if the first $\lambda$ mutations to the duplicate gene yield compatible residues; thus, we ignore that possibility.)

If the probability of an event is $P$, then of course on average $1/P$ opportunities will be required before the event occurs. Thus, to produce an MR feature in our model will require an average number of opportunities equal to the inverse of the probability discussed earlier, or

$$(1+\rho)^{\lambda}\left(\frac{\lambda^{\lambda}}{\lambda!}\right).$$

At steady state, the number of opportunities to produce an MR function in a given time period in a population will be equal to the number of point mutations that occur in the potential MR site across the population—that is, to the time multiplied by the mutation rate per nucleotide $\nu$, the number of nucleotide positions $\lambda$ that must mutate to compatible residues, and the population size $N$—that is, equal to $N\nu\lambda t$. To produce a gene with $\lambda$ compatible mutations, the incompatible residue in a gene with $\lambda - 1$ compatible mutations has to be mutated, so that the time to produce an MR function with $\lambda$ compatible sites will be proportional to the degree of saturation of the system with genes containing $\lambda - 1$ compatible sites. However, as exemplified by Figure 2, our model does not start at steady state; it starts with all sites in the incompatible state. Thus, the time required to produce an event will also depend on the degree to which the system has approached steady state, as follows. If the degree of saturation for one compatible site is in general $S$, then the degree of saturation for $n$ compatible sites is $S^n$.

Thus, the degree of saturation with $\lambda - 1$ compatible sites at any given time is equal to the degree of saturation given in equation 2 raised to the $\lambda - 1$ power. Because the degree of saturation changes in time, to find the total number of opportunities for producing an MR feature, this value must be integrated over time.

These considerations can be combined to yield a quantitative description of the behavior of the model with time. The expected average time $T_f$ to the first occurrence of an MR feature for a population of duplicate genes initially in a uniform state, needing $\lambda$ positions mutated to acquire the MR feature, and with a ratio $\rho$ of null-to-compatible mutations, can be evaluated by equation 3.

$$N\nu\lambda \int_0^{T_f} \left(1 - \exp\left(\frac{-\nu t(2+\rho\lambda)}{1+\rho}\right)\right)^{(\lambda-1)} dt = (1+\rho)^{\lambda} \left(\frac{\lambda^{\lambda}}{\lambda!}\right) \quad (3)$$

The right-hand side of equation 3 is the inverse of the probability discussed earlier. The left-hand side gives the number of opportunities for production of the MR feature in the nonequilibrium system starting with no nucleotide positions in compatible states. The preintegral term of the left-hand side of the equation, $N\nu\lambda$, is the number of point mutations occurring in the population per unit time at steady state. The integrand of equation 3, which is the numerator from the right-hand side of equation 2 raised to the power of $\lambda - 1$, is the degree of saturation of the system with "preselectable" mutants—that is, mutants that are one step from being selectable, with $\lambda - 1$ sites in the compatible state.

Figure 3 shows the result of simulations in which the number of sites $\lambda$ in an MR feature was varied along with the ratio $\rho$ of null-to-compatible mutations and the haploid population size $N$. As can be seen, the curves generated by equation 3 match the results of the simulations very closely for a wide range of values of $N$, $\rho$, and $\lambda$.

### *The effect of selection*

The simulations shown in Figure 3 examined the number of generations required to produce just the first occurrence of

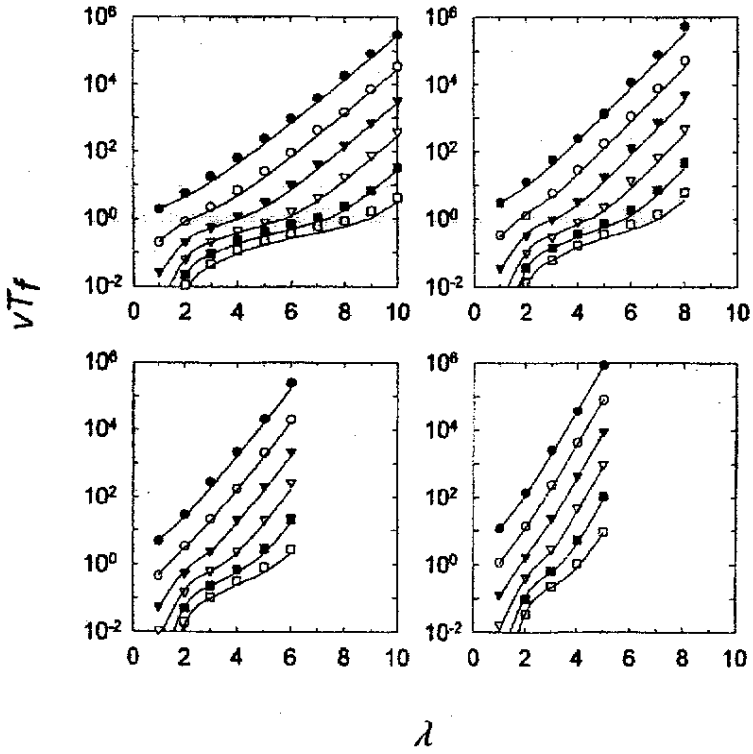


**Figure 3.** Normalized time (generations) to first appearance ($\nu T_f$) versus number of loci $\lambda$ required to be changed to yield the multiresidue (MR) feature. In all cases, the curves are determined from equation 3. $\nu = 0.01$. Reproduction was deterministic. Filled circles, $N = 1$; open circles, $N = 10$; filled inverted triangles, $N = 100$; open inverted triangles circles, $N = 1000$; filled squares, $N = 10{,}000$; open squares, $N = 100{,}000$. (*Upper left*) $\rho = 1$; (*upper right*) $\rho = 2$; (*lower left*) $\rho = 4$; (*lower right*) $\rho = 10$. Each point is the average of 100 repetitions.

an MR feature in a population. However, beneficial mutations are frequently lost from a population by stochastic processes before fixation (Kimura 1983). In Figure 4, we present the results of simulations which determine the time to fixation $T_{fx}$ of the MR feature in the population as a function of the strength of the selection coefficient $s$. The simulation results are well fit by equation 4.

$$N\nu\lambda\int_0^{T_{fx}}\left(1-\exp\left(\frac{-\nu t(2+\rho\lambda)}{1+\rho}\right)\right)^{(\lambda-1)}dt=\frac{(1+\rho)^{\lambda}}{2s}\left(\frac{\lambda^{\lambda}}{\lambda!}\right) \quad (4)$$

Equation 4 is a minor modification of equation 3, where the right-hand side of the equation is divided by twice the selection coefficient. This result follows from the dependence of the fixation probability on the selection coefficient (Li 1997).

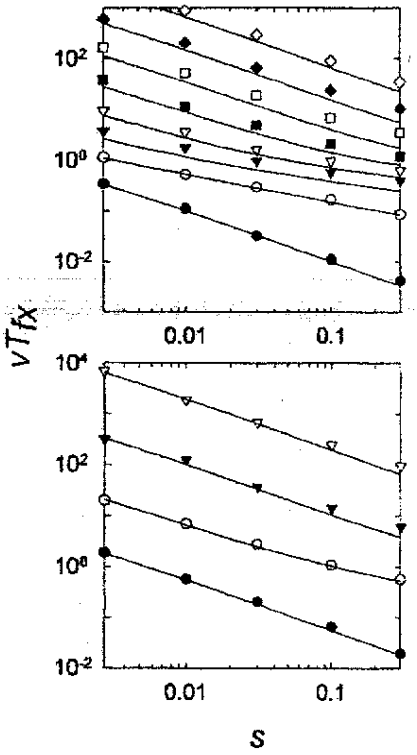


Figure 4. Normalized time (generations) to fixation ($\nu T_{fx}$) versus the selection coefficient $s$. In all cases, the curves are determined from equation 4. Reproduction was stochastic. $N = 1000$; $\nu = 0.01$–$0.0001$. Each point is the average of 100 repetitions. (*Top*) $\rho = 1$. Filled circles, $\lambda = 1$; open circles, $\lambda = 2$; filled inverted triangles, $\lambda = 3$; open inverted triangles circles, $\lambda = 4$; filled squares, $\lambda = 5$; open squares, $\lambda = 6$; filled diamonds, $\lambda = 7$; open diamonds, $\lambda = 8$. (*Bottom*) $\rho = 10$. Filled circles, $\lambda = 1$; open circles, $\lambda = 2$; filled inverted triangles, $\lambda = 3$; open inverted triangles, $\lambda = 4$.

*Pre-equilibration of the population*

Thus far, the starting point for the model has been a uniform population in which all genes are initially present as exact duplicates of the parent gene. Mutations then begin to accumulate and the program immediately starts to check for the presence of the MR feature, simulating the presence of selective pressure from the start. However, a different situation can also be considered, in which the duplicate gene begins to undergo mutation, but selective pressure arises only at a later time, perhaps as a result of environmental changes. In that case, the population of duplicate genes will be at least part of the way toward its steady-state frequency before selection affects the population. This can be modeled in the simulation by neglecting to check for the presence of the MR feature, treating it as a neutral property, until a predetermined number of generations have passed.

Figure 5 shows the result of simulations in which all duplicate genes began in a uniform state, identical to the parent gene, but the population was allowed to undergo mutation and reproduction for varying periods of time before starting to check for the MR feature. It can be seen that as the length of the pre-equilibration period increases, the average time from the start of selection to observation of the duplicate gene coding for the new MR feature decreases for population sizes, where, at steady state in the absence of selection, at least one duplicated gene with the feature is expected to already be present in the population, that is, where the population size is greater than the inverse of the probability of producing the MR feature, $N > (1+\rho)^{\lambda}(\lambda^{\lambda}/\lambda!)$. In Figure 5, this occurs at $\lambda \leq 5$. For the case where $N < (1+\rho)^{\lambda}(\lambda^{\lambda}/\lambda!)$ (at $\lambda \geq 6$ in Fig. 5), however, the expected time is essentially unaffected by pre-equilibration of the population. Because it follows from equation 3 that $N < (1+\rho)^{\lambda}(\lambda^{\lambda}/\lambda!)$, when $\nu$ times the evaluated integral is >1, then $T_f$ will be substantially unaffected by pre-equilibration when $T_f \geq 1/\nu$.

## Discussion

*The model and its limits*

Some features of proteins, such as disulfide bonds and ligand binding sites, which here we call MR features, are composed of multiple amino acid residues. As Li (1997) points out, the evolutionary origins of such features must have involved multiple mutations that were initially neutral with respect to the MR feature. We have attempted to model such a process. In doing so, one might examine a number of possible routes to an MR feature, for example, looking at a unique gene that is under selective constraints, or looking at mutations caused by insertions and deletions or recombination in a duplicate gene. Our model is restricted to the development of MR features by point mutation in a dupli-

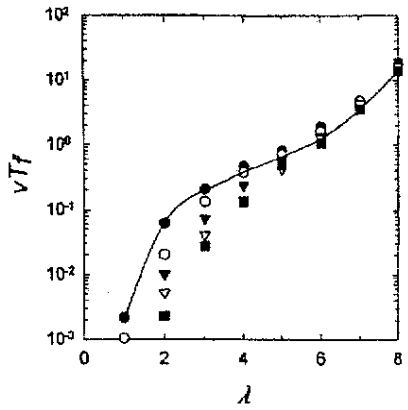


**Figure 5.** Effect of pre-equilibration of the population on normalized time (generations) to first appearance ($\nu T_f$) versus number of loci $\lambda$ required to be changed to yield the MR feature. $N = 1000$; $\nu = 0.001$; $\rho = 1$. Each point is the average of 100 repetitions. The curve is determined from equation 3. Reproduction was deterministic. The simulation was pre-equilibrated (that is, the population was subject to mutation and reproduction without checking for the appearance of the multiresidue (MR) feature, regarding it as neutral) for filled circles, 0 generations; open circles, $0.1/\nu$ generations; filled inverted triangles, $0.3/\nu$ generations; open inverted triangles, $1/\nu$ generations; filled squares, $3/\nu$ generations.

cated gene. We strongly emphasize that results bearing on the efficiency of this one pathway as a conduit for Darwinian evolution say little or nothing about the efficiency of other possible pathways. Thus, for example, the present study that examines the evolution of MR protein features by point mutation in duplicate genes does not indicate whether evolution of such features by other processes (such as recombination or insertion/deletion mutations) would be more or less efficient.

There are several reasons, both practical and theoretical, for examining this limited model. First, as mentioned earlier, gene duplication is considered to be a major route to evolutionary novelty (Ohno 1970; Lynch and Conery 2000; Wagner 2001; Chothia et al. 2003) and therefore it is important to explore its potential in regard to MR features. Second, a duplicated gene can be considered to be largely free of the effects of purifying selection (but see following) and therefore selective effects, which are difficult to estimate, can be ignored, simplifying the task at hand. Third, point mutations are well-defined events, where transitions occur among a limited set of states. In contrast, insertions and deletions vary in size and composition, making them difficult to model for our purposes. Thus, we confine our model of the development of MR features to what we consider to be the conceptually simplest and computationally most tractable route, of point mutations in a duplicated gene that is free of purifying selection.

Is the assumption of the selective neutrality of duplicated genes either a realistic or a useful one? On the one hand, the assumption appears not to be correct in at least some situations. For example, although the vast majority of neutral duplicated genes are expected to result in null alleles, studies of polyploid organisms showed that more duplicate genes survived over long periods of time than expected (Ferris and Whitt 1977, 1979; Hughes and Hughes 1993; White and Doebley 1998). This has provoked the suggestion that gene dosage effects in polyploids might slow the decay of duplicate gene copies and that more duplicates may be preserved than expected by the process of subfunctionalization, where a gene with two or more functions duplicates and each copy subsequently loses one of the functions and then goes on to specialize in the preserved function (Force et al. 1999; Lynch et al. 2001). Although the assumption of the selective neutrality of duplicated genes does not fit data from some polyploid species (Ferris and Whitt 1977, 1979; Hughes and Hughes 1993; White and Doebley 1998), it may yet be a good model for individual gene duplication events (Lynch and Conery 2000). In support of this view, recent studies have shown that genes that have been recently duplicated seem to be under relaxed selection, as indicated by the similar number of synonymous and nonsynonymous mutations they have acquired (Lynch and Conery 2000; Kondrashov et al. 2002).

On the other hand, it should be emphasized that the utility of the idealized model presented here—where there is no selective effect from duplicate genes or from intermediate states of the gene until the MR feature is completely in place in a gene and where the only mutagenic process considered is point mutations—is not dependent on a comprehensive accounting for all relevant biological processes. Rather, its usefulness lies in its ability to indicate when processes in addition to those described in the model are required to account for a feature. If the development of an MR feature by means of point mutation in an ideal, neutral, duplicated gene would require unrealistically large population sizes or unrealistically long times, then one can conclude that other factors (such as recombination, selection of intermediate states, and/or other factors) must be examined to account for the feature. Because neutral gene duplication and point mutation is often invoked to account for complex features of proteins, it would be useful to have a quantitative understanding for what such scenarios would entail in order to assess their reasonableness.

In our simulations, the model starts in a uniform initial state, with the population already in possession of $N$ exact duplicates of the parent gene. This, of course, is biologically unrealistic but can be considered to approximate the end result of either of two processes: (1) the spread of a duplicate gene through a population by random drift until it is fixed or (2) the occurrence of a phylogenetic branching point, where after the branch point, a small population that is homogeneous with respect to the duplicate gene expands to a population size $N$. Although mutations will occur in

copies of the duplicate gene during the period of either drift of the gene or expansion of the population, there will be fewer mutations—and thus fewer opportunities to produce the MR feature—than in a population already at size $N$, each with on average one copy of the duplicate gene, for the same period of time. In either case, the time to reach the initial state is neglected, so the time obtained from the simulations can be considered to be an underestimate of the time to fixation $T_{fx}$ of the MR feature. Although we envision each organism of the population as having one duplicate gene per haploid genome, because recombination is disallowed and each duplicate accumulates mutations independently, it does not affect the model (as represented by equation 4) if there is variation in copy number of the duplicate gene in organisms, as long as the total number of duplicate gene copies in the population is $N$.

Figure 3 shows that the results of the simulation closely match those predicted from equation 3, which gives us confidence to extrapolate to biologically realistic values of the parameters of the equation. The curves in Figure 3 exhibit two regions: (1) a nonlinear region at larger population sizes and/or smaller numbers of sites and (2) a linear region at smaller population sizes and/or higher numbers of loci. These regions represent, respectively, (1) the situation where in the absence of selection for the MR feature the steady-state population would be expected to contain one or more copies of the duplicate gene with an MR feature [that is, where $N > (1 + \rho)^{\lambda}(\lambda^{\lambda}/\lambda!)$] and (2) the situation where in the absence of selection, the population on average at steady state is not expected to contain a copy with the MR feature [that is, where $N < (1 + \rho)^{\lambda}(\lambda^{\lambda}/\lambda!)$].

The expected time for the nonlinear region largely reflects the amount of time necessary for the population to approach steady state. This time is on the order of the inverse of the rate of point mutation and is relatively insensitive to either the number of loci involved in the MR feature or the population size, varying inversely with only the $\lambda^{th}$ root of $N$ (see Appendix 2). Thus, the ability to decrease the time required to produce an MR feature much below $1 / \nu$ by increasing population size is greatly constrained by the nonlinearity of the model, reflecting the slow equilibration of the population when multiple mutations are required.

As shown in Figure 4, the effect of changes in the selection coefficient on the behavior of the model are closely fit by equation 4. It should be noted that the time calculated from equation 4 reflects the average time required simply to produce the MR mutant that will go on to become fixed in the population; it does not explicitly include the time required for the mutation to spread and become fixed in the population once it has been produced. The close fit of the simulation results of Figure 4—which does include both the time to produce the MR mutation that will be fixed plus the time required for the mutation to spread through the population to fixation—to the curve predicted from equation 4 emphasizes the fact that the timescale for fixation of the mutation is negligible compared with the timescale required to produce the mutation that will go on to become fixed.

As shown in Figure 5 for the nonlinear region, if the population has been accumulating mutations for a period of time before selection for the MR feature is applied (perhaps representing a population approaching steady state where the environment then changes, making a feature selectable that previously had been neutral), then the expected time, measured from the start of selection to the appearance of the MR feature, decreases. On the other hand, as also shown in Figure 5, for situations where the population is not expected to have a copy of the duplicate gene with the MR feature at steady state (in Figure 5, for $\lambda \geq 6$), then the expected time to its fixation is essentially unaffected by pre-equilibration of the population. This is the case whenever $T_f \geq 1 / \nu$. It should be noted that pre-equilibration explicitly allows for the occurrence of rare, "lucky" alleles whose sequence is closer to that of the MR feature than is the sequence of the starting, predominant gene. Such rare alleles could thus be poised to give rise to the MR feature in perhaps one or two steps. The result shown in Figure 5—that, for $\lambda \geq 6$, pre-equilibration has no effect on $T_f$—demonstrates that on average the opportunity for the serendipitous occurrence of rare alleles does not alter the expected time.

### *Estimation of $T_{fx}$ for several cases*

Estimated values for parameters of our model can be garnered from the literature. Drake et al. (1998) estimate the deleterious mutation rate to be about 0.2–2.0 per generation per effective genome size of $10^8$ bp for a variety of multicellular organisms, both vertebrate and invertebrate. We use that number to approximate the effective nucleotide point mutation rate per generation $\nu$ in coding regions to be on the order of $10^{-8}$. Lynch and Conery (2000) calculate the rate of duplication of a given gene to be 0.01 per million years—in other words, $10^{-8}$ per year, which for our purposes we consider to be roughly equal to the estimated nucleotide point mutation rate (Lynch and Conery 2000; see also the discussion of that work [Long and Thornton 2001; Lynch and Conery 2001; Zhang et al. 2001]). Although here we assign single values to the parameters, one must keep in mind that there is significant uncertainty in estimating them and that the rates may vary with time, species, region of the genome, and other factors.

An estimate of $\rho$ can be inferred from studies of the tolerance of proteins to amino acid substitution. Although there is variation among different positions in a protein sequence, with surface residues in general being more tolerant of substitution than buried residues, it can be calculated that on average a given position will tolerate about six different amino acid residues and still maintain function

(Reidhaar-Olson and Sauer 1988, 1990; Bowie and Sauer 1989; Lim and Sauer 1989; Bowie et al. 1990; Rennell et al. 1991; Axe et al. 1996; Huang et al. 1996; Sauer et al. 1996; Suckow et al. 1996). Conversely, mutations to an average of 14 residues per site will produce a null allele, that is, one coding for a nonfunctional protein. Thus, in the coding sequence for an average-sized protein domain of 200 amino acid residues, there are, on average, 2800 possible substitutions that lead to a nonfunctional protein as a result of direct effects on protein structure or function. If several mutations are required to produce a new MR feature in a protein, then ρ is roughly of the order of 1000. This value for ρ is on the low end used by Walsh (1995), who considered values for ρ up to $10^5$. (Walsh, however, defined ρ as the ratio of advantageous-to-null mutations—the inverse of our definition.)

It should be emphasized that the value of ρ is not the ratio of mutations to an organismal genome that would be lethal to those that would be mildly deleterious. Rather, it is the ratio of the number of mutations that would inactivate a typical protein to the number that would lead to a new MR feature for that particular protein. Many genes can be silenced with small or moderate ill effect on the organism (for example, the gene for myoglobin can be inactivated in mice with little ill effect in adult mice [Garry et al. 1998, 2003; Meeson et al. 2001]). However, if a mutation inactivates a protein, then it is counted in the model as a null mutation for purposes of calculating ρ, whether or not it may have severe phenotypic effects. The best estimate for this number comes not from studies of mutations in organisms, but rather from studies of the tolerance of specific proteins to mutation (Reidhaar-Olson and Sauer 1988, 1990; Bowie and Sauer 1989; Lim and Sauer 1989; Bowie et al. 1990; Rennell et al. 1991; Axe et al. 1996, 1998; Huang et al. 1996; Sauer et al. 1996; Suckow et al. 1996).

The uncertainties involved in estimating ρ should be kept in mind. On the one hand, although as just discussed, studies selecting for activity of mutant proteins show most substitutions to reduce function below that required for a biological assay, a study searching for inactivating mutations to the autotoxic ribonuclease barnase showed that comparatively few substitutions reduced activity to that of uncatalyzed reactions (Axe et al. 1998). This consideration may lower the estimate of ρ. On the other hand, duplicate genes might also be lost by processes other than point mutation, such as deletion or recombination. Additionally, null mutations in the coding sequence or flanking sequences might occur because of indirect effects such as, for example, altering the stability of the mRNA. These considerations might effectively increase the value of ρ.

Figure 6 uses equation 4 and the values for ν and ρ estimated earlier to plot the expected time in generations to the fixation of an MR protein feature for populations of different sizes. In addition, we use a value of 0.01 for the selection coefficient $s$. Figure 6 shows that the fixation of specific MR features by point mutation in duplicated genes is a long-term phenomenon that requires populations of considerable size. For example, consider a case where three nucleotide changes must be made to generate a novel feature such as a disulfide bond. In that instance, Figure 6 shows that a population size of approximately $10^{11}$ organisms on average would be required to give rise to the feature over the course of $10^8$ generations, and this calculation is unaffected by pre-equilibration of the population in the absence of selection. To produce the feature in one million generations would, on average, require an enormous population of about $10^{17}$ organisms, although this number would change if the population had pre-equilibrated in the absence of selection.

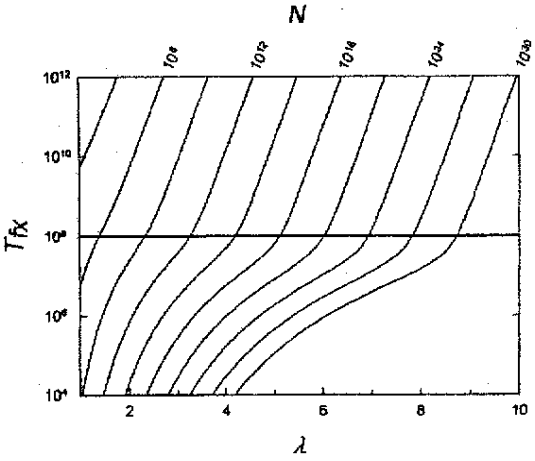


**Figure 6.** Time to fixation $T_{fx}$ versus number of loci λ required to be changed to yield the multiresidue (MR) feature. $\nu = 10^{-8}$; $\rho = 1000$; $s = 0.01$. Values for population sizes $N$ are given across the *top* axis. In all cases the curves are determined from equation 4. A line is drawn across the figure at $T_{fx} = 1/\nu$, which is $10^8$ generations. *Above the line*, values for $T_{fx}$ are essentially unaffected by pre-equilibration of the population in the absence of selection.

For features requiring more participating residues, the expected population sizes are even larger. As Li (1997) noted, the binding site for diphosphoglycerate in hemoglobin requires three residues. The population size required to produce an MR feature consisting of three interacting residues by point mutation in a duplicated gene initially lacking those residues would depend on the number of nucleotides that had to be changed—a minimum of three and a maximum of nine. If six mutations were required then, as indicated by Figure 6, on average a population size of ~$10^{22}$ organisms would be necessary to fix the MR feature in $10^8$ generations, and a population of ~$10^{30}$ organisms would be expected to fix the mutation in one million generations. In a recent in vitro study intended to mimic evolution, a re-

combinant amphioxus insulin-like peptide was altered by site-directed mutagenesis at seven nucleotide positions to contain five altered amino acid residues that would allow interaction with mammalian insulin receptor (Guo et al. 2002). In order for such a process to occur in vivo by gene duplication and point mutation within a hundred million generations would be expected on average to require $>10^{25}$ organisms.

Such numbers seem prohibitive. However, we must be cautious in interpreting the calculations. On the one hand, as discussed previously, these values can actually be considered underestimates because they neglect the time it would take a duplicated gene initially to spread in a population. On the other hand, because the simulation looks for the production of a *particular* MR feature in a *particular* gene, the values will be overestimates of the time necessary to produce *some* MR feature in *some* duplicated gene. In other words, the simulation takes a prospective stance, asking for a certain feature to be produced, but we look at modern proteins retrospectively. Although we see a particular disulfide bond or binding site in a particular protein, there may have been several sites in the protein that could have evolved into disulfide bonds or binding sites, or other proteins may have fulfilled the same role. For example, Matthews' group engineered several nonnative disulfide bonds into lysozyme that permit function (Matsumura et al. 1989). We see the modern product but not the historical possibilities.

We should also notice which parameters the model is particularly sensitive to and which not. The model is least sensitive to the point mutation rate $\nu$ and the selection coefficient $s$ because both of those appear only as linear terms in equation 4. Thus, for example, if we consider an organism where the point mutation rate is increased by a factor of $10^3$, then the numbers calculated from equation 4 will decrease by only that factor. For the case discussed earlier in which six nucleotide changes were required, the population size needed to fix the feature in $10^8$ generations would then decrease from $10^{22}$ to just $10^{19}$.

The model is more sensitive to the value of $\rho$, because $\rho$ appears with an exponent in equation 4. If $\rho$ were less by a factor of 10 (100 instead of 1000), then the population size needed to fix the feature in the preceding example in $10^8$ generations would decrease from $10^{22}$ to $10^{16}$. The number of possible null mutations—the numerator of $\rho$—arises from basic considerations of protein structure so that it is unlikely to vary significantly. The number of possible compatible mutations $\lambda$—the denominator of $\rho$—is more difficult to estimate. However, the value of one thousand that we use for $\rho$ in Figure 6 is conservative compared with the range of values used by other workers (Walsh 1995). It should be noted that as $\lambda$ becomes larger, the number of possible null mutations—and thus implicitly the length of the gene—must increase to maintain a constant value of $\rho$.

The model is most sensitive to the value of $\lambda$—the number of loci that must mutate before a new MR function occurs—which appears as an exponent in equation 4. If in the case just mentioned, because of the particular initial sequence of the parent gene, either three or nine nucleotide changes were necessary instead of six, then the population sizes required to fix the feature in $10^8$ generations would vary from $10^{11}$ to $10^{31}$ organisms. The dependence on $\lambda$ may encourage speculation that perhaps MR mutations could develop by point mutation in duplicate genes if the parent gene giving rise to the duplication were serendipitously poised to lead to the new feature with only one mutation in the precursor gene. Although this is certainly possible, it is unlikely to be the general case. As one example, Li (1997) has argued that the precursor to modern hemoglobins that can bind diphosphoglycerate did not have any of the three amino acid residues involved in the interaction. As shown in Figure 5, for the average case, pre-equilibration, which allows for the occurrence of rare, fortunate alleles, does not affect the expected time $T_f$ in the linear portion of the curve.

The lack of recombination in our model means it is most directly applicable to haploid, asexual organisms. Nonetheless, the results also impinge on the evolution of diploid sexual organisms. The fact that very large population sizes—$10^9$ or greater—are required to build even a minimal MR feature requiring two nucleotide alterations within $10^8$ generations by the processes described in our model, and that enormous population sizes are required for more complex features or shorter times, seems to indicate that the mechanism of gene duplication and point mutation alone would be ineffective, at least for multicellular diploid species, because few multicellular species reach the required population sizes. Thus, mechanisms in addition to gene duplication and point mutation may be necessary to explain the development of MR features in multicellular organisms.

Although large uncertainties remain, it nonetheless seems reasonable to conclude that, although gene duplication and point mutation may be an effective mechanism for exploring closely neighboring genetic space for novel functions, where single mutations produce selectable effects, this conceptually simple pathway for developing new functions is problematic when multiple mutations are required. Thus, as a rule, we should look to more complicated pathways, perhaps involving insertion, deletion, recombination, selection of intermediate states, or other mechanisms, to account for most MR protein features.

## Materials and methods

A duplicated gene in a population was represented by an array of integer elements that could take the values of either zero or one. The number of elements $\lambda$ in the array corresponded to the number

of nucleotide positions that would have to mutate in a particular gene to yield a hypothesized MR protein feature. In all cases, we begin the simulation in a uniform initial state, with $N$ duplicate copies of the parent gene in the population, represented by $N$ identical arrays. This simplification of starting with the duplicate gene already fixed in the population ignores the time needed for the duplicate copy to initially spread in the population; thus the average times we calculate from this model can be considered underestimates of the time for fixation of a gene with a new MR function. All elements of the array were initially set to a value of one, which represented the initial, incompatible state of the position, which could not contribute to the MR feature. Each position in an array was then allowed to mutate sequentially with a probability set by the mutation rate $\nu$. A value of zero represented the state that could potentially contribute to an MR feature. Back mutations were permitted, so that a position with a value of zero could revert to a value of one. The equal rates of forward and backward point mutations should not be confused with the very different rates at which a gene will acquire null mutations versus acquiring a new, selectable MR feature.

After each step in which a mutation occurred at an array position, a further probabilistic step was taken to simulate the possible occurrence of one or more null mutations elsewhere in the gene. With probability $1/(1+\rho)$, where $\rho$ is the ratio of null-to-compatible mutations in the gene (neutral mutations are ignored), the gene was considered to be free of null mutations and continued in the simulation. With probability $\rho/(1+\rho)$ the gene was deemed to have suffered one or more null mutations at positions not explicitly represented in the array and consequently to have become a pseudogene. In this case, the array was replaced in the population by a new array in which all loci were again set to one. This is intended to simulate replacement of the nonfunctional duplicate gene by a new duplication of the original gene, whose sequence is considered to remain constant under selection. Checking for null mutations only when mutations occur at an array position has the intended effect of making the gene duplication rate similar to the rate of point mutation $\nu$. It has recently been shown that those two rates are in fact similar (Lynch and Conery 2000). It should be noted that the model purposely does not replace the duplicate gene immediately whenever a null mutation would occur anywhere in the gene, rather than waiting for one of the $\lambda$ array sites to mutate, because that would have the effect of making the gene duplication rate much faster than it is estimated to be.

After the mutation step, the population was checked for the number of selectable organisms—those whose array elements all had a value of zero. Arrays in which some but not all elements were in a compatible state had no advantage. (This models MR features where, by hypothesis, the selectable feature does not exist until all contributing amino acids are in the correct state.) For most simulations, the run was halted when the first selectable array was discovered and the time in generations to the first occurrence of the selectable MR mutant recorded. For other runs, the simulation was continued and selection was applied at the reproduction step. In these cases, the simulation was continued until >50% of the population carried the MR feature, which was then considered to be "fixed" in the population, and the time to fixation recorded. The time for the selectable MR mutation to spread is generally much less than the time for it to be produced by the population.

After each array was subjected to the mutation step, the next generation was populated, either by deterministic reproduction or by simulated stochastic reproduction. In deterministic reproduction, the next generation was taken clonally from the previous; that is, the composition of the next generation was identical to the previous generation after the mutation step. In stochastic reproduction, the subsequent generation was populated by copying randomly chosen arrays, with or without selection, from the previous generation until the subsequent generation was fully populated.

In some simulations, the process was "prerun" for a selected number of generations, undergoing mutation and reproduction but not selection. This was done to model populations that had to varying degrees approached steady state with respect to the occurrence of the MR mutation in the population in the absence of selection for it.

All equations were evaluated using *Mathematica*.

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## Appendix 1

Consider a population in which the number of duplicated genes that has none of the necessary mutations to produce an MR feature is $n_0$, the number that has one required mutation is $n_1$, the number with two required mutations is $n_2$, and so forth. As in the text, $\nu$ is the mutation rate per nucleotide per generation, $\rho$ is the ratio of possible null mutations to compatible mutations in a gene, and $\lambda$ is the number of initially incompatible nucleotide loci in a duplicate gene that must be changed to form the selectable, MR feature. Also as in the text, genes suffering a null mutation are presumed to be replaced by a new duplicate of the original gene, with all loci in the incompatible state. Then we can write:

$$\frac{\partial n_0}{\partial t} = -\lambda\nu\alpha n_0 + \nu\alpha n_1 + \nu\beta n_1 + \nu\beta n_2 + \ldots + \nu\beta n_{\lambda-1} \quad (5)$$

$$\frac{\partial n_1}{\partial t} = \lambda\nu\alpha n_0 - \nu\alpha n_1 - \nu\beta n_1 - (\lambda-1)\nu\alpha n_1 + 2\nu\alpha n_2 \quad (6)$$

$$\frac{\partial n_2}{\partial t} = (\lambda-1)\alpha n_1 - 2\nu\alpha n_2 - \nu\beta n_2 - (\lambda-2)\nu\alpha n_2 + 3\nu\alpha n_3 \quad (7)$$

$$\vdots$$

$$\frac{\partial n_\lambda}{\partial t} = \nu\alpha n_{\lambda-1} \quad (8)$$

where

$$\alpha \equiv \frac{1}{1+\rho}$$

and

$$\beta \equiv \frac{\rho\lambda}{1+\rho}$$

Terms representing processes in which an additional compatible mutation is gained without a null mutation first occurring are multiplied by the factor $\lambda - m$, where $m$ is the number of compatible mutations a gene has already acquired, to account for the decreasing number of sites that are available for potentially beneficial mutation, and terms representing processes in which a compatible mutation is lost without a null mutation first occurring are multiplied by $m$ to account for the increasing number of sites that can revert. Equation 8 represents the transition to an absorbing state that is under selection, where all required mutations necessary for the new MR feature are present. Once in the selectable state, the gene is presumed not to leave by mutation to another state. (This is similar to the situation described by equation 3, where the time to just the first appearance of an MR feature is estimated, which thus does not allow for back mutation of the gene with the MR feature.)

If $\rho \gg 1$, then $n_0 \gg n_1 \gg n_2 \ldots$ and $\beta \gg \alpha$. In this limit, equations 5–8 become simply:

$$\frac{\partial n_0}{\partial t} = -\lambda\nu\alpha n_0 + \nu\beta n_1 \tag{9}$$

$$\frac{\partial n_1}{\partial t} = \lambda\nu\alpha n_0 - \nu\beta n_1 \tag{10}$$

$$\frac{\partial n_2}{\partial t} = (\lambda-1)\nu\alpha n_1 - \nu\beta n_2 \tag{11}$$

$$\frac{\partial n_\lambda}{\partial t} = \nu\alpha n_{\lambda-1} \tag{12}$$

These can be solved in sequence by successive approximations. For equation 10, we approximate $n_0 = N$ (the total population size), which for initial condition $n_1 = 0$ gives:

$$\frac{\partial n_1}{\partial t} = \lambda\nu\alpha N - \nu\beta n_1 \tag{13}$$

which has the solution

$$n_1 = N\lambda\frac{\alpha}{\beta}(1 - e^{-\nu\beta t}). \tag{14}$$

Using this solution for $n_1$ in the next equation, with initial condition $n_2 = 0$, we have

$$n_2 = N\lambda(\lambda - 1)\left(\frac{\alpha}{\beta}\right)^2(1 - e^{-\nu\beta t})^2 \tag{15}$$

Therefore, using the same approximation successively, we obtain

$$n_{\lambda-1} = N(\lambda)!\left(\frac{\alpha}{\beta}\right)^{\lambda-1}(1 - e^{-\nu\beta t})^{\lambda-1}. \tag{16}$$

Last, we integrate equation 16 to get $n\lambda$. Setting $n\lambda = 1$ (the first appearance of the MR feature) yields

$$1 = \int_0^{T_f}(\lambda!)\nu\alpha N\left(\frac{\alpha}{\beta}\right)^{\lambda-1}(1 - e^{-\nu\beta t})^{\lambda-1}\,dt, \tag{17}$$

or

$$N\nu\lambda\int_0^{T_f}(1 - e^{-\nu\beta t})^{\lambda-1}\,dt = \frac{\lambda(1+\rho)(1+\lambda\rho)^{\lambda-1}}{\lambda!}. \tag{18}$$

When $\rho$ is small (<10), the values of $T_f$ given by equation 18 do not give a good match to the results of the simulations, which are nonetheless closely matched by equation 3 in the text. When $\rho$ is large ($\geq$10), however, which will be the case in biologically realistic situations, then equation 18 is approximately equal to equation 3 and both equations closely match the results of the simulations because, for large $\rho$, for the exponent of the integrand on the left-hand sides of the equations

$$\frac{2+\rho\lambda}{1+\rho} \approx \frac{\rho\lambda}{1+\rho} \approx \lambda$$

and for the right-hand sides of the equations

$$(1+\rho)^\lambda\left(\frac{\lambda^\lambda}{\lambda!}\right) \approx \frac{\lambda(1+\rho)(\lambda\rho)^{\lambda-1}}{\lambda!} \approx \frac{\rho^\lambda\lambda^\lambda}{\lambda!}.$$

## Appendix 2

In the limit of $T_f \ll 1/\nu\lambda$, the left-hand sides of both equation 3 and equation 18 are proportional to $(T_f)\lambda$. This is seen in the following:

$$\int_0^{T_f}(1 - e^{-\gamma t})^{(\lambda-1)}\,dt \approx \int_0^{T_f}(1-(1-\gamma t))^{(\lambda-1)}\,dt \tag{19}$$

$$= \gamma^{(\lambda-1)}\int_0^{T_f} t^{(\lambda-1)}\,dt \tag{20}$$

$$= \frac{\gamma^{(\lambda-1)}}{\lambda}T_f^\lambda \tag{21}$$

where $\gamma = \nu\lambda$ for both equations in the limit $\rho \gg 1$. In this limit, equations 3 and 18 therefore become

$$N\nu\lambda\left(\frac{(\nu\lambda)^{\lambda-1}}{\lambda}T_f^\lambda\right) = \frac{\rho^\lambda\lambda^\lambda}{\lambda!} \tag{22}$$

which implies

$$\nu T_f \approx \left(\frac{\rho^\lambda\lambda^\lambda}{\lambda!N}\right)^{1/\lambda}. \tag{23}$$

where we have used the approximations of Appendix 1 for the case of $\rho \gg 1$. The limit $T_f \ll 1/\nu\lambda$ therefore corresponds to the limit $N \gg (1+\rho)^\lambda(\lambda^\lambda/\lambda!)$, or $\rho \ll N^{1/\lambda}$.

For $\rho \ll N^{1/\lambda}$, then, the time required to produce a selectable state is inversely proportional to the $\lambda^{th}$ root of the population size, that is, $T_f \propto N^{-(1/\lambda)}$.

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# Exhibit 11

article on evolutionary potential
by Barry Hall

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# In Vitro Evolution Predicts that the IMP-1 Metallo-β-Lactamase Does Not Have the Potential To Evolve Increased Activity against Imipenem

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**In vitro evolution was used to predict whether the IMP-1 metallo-β-lactamase has the potential to evolve an increased ability to confer resistance to imipenem. Screening of eight libraries containing $9.8 \times 10^6 \pm 1.4 \times 10^6$ (mean ± standard error) variants per library, with an average of 1.2 mutations per variant, detected no increased resistance to imipenem. The results predict, with >99.9% confidence, that even under intense selection the IMP-1 β-lactamase will not evolve to confer increased resistance to imipenem.**

Carbapenems are among the most potent agents for treatment of gram-negative bacterial infections (9, 12) and are hydrolyzed by a wide variety of metallo-β-lactamases (9). Widespread clinical use of carbapenems (8, 12) has led to several reports of resistance associated with the presence of metallo-β-lactamases (7). The IMP family of metallo-β-lactamases is a particular source of concern, because the IMP enzymes are typically plasmid-borne and typically found in integron cassettes (5, 10). IMP genes are therefore easily transferred among diverse bacterial species. A number of researchers have advised careful clinical use to prevent proliferation of carbapenem-resistant strains that produce metallo-β-lactamases (7, 12). Yano et al. (12) recently reported that IMP-6, which differs from IMP-1 by a single amino acid substitution, increases the MIC of meropenem 128-fold but does not increase the resistance to imipenem. If the rapid evolution of the class A extended-spectrum β-lactamases is typical, then we should indeed be concerned about the evolution of metallo-β-lactamases in response to the clinical use of imipenem and other carbapenems.

Instead of assuming that metallo-β-lactamases will evolve rapidly, it would be highly desirable to accurately predict their evolution in response to carbapenem selection.

The Barlow-Hall in vitro evolution model has been shown to accurately mimic the natural evolution of the TEM β-lactamases (4) and has been used to predict that both the class A TEM and class C CMY-2 β-lactamases will soon evolve to provide high levels of resistance to cefepime (2, 3). Here I have used that method to predict the evolution of the IMP-1 β-lactamase in response to clinical selection with imipenem.

The $bla_{IMP-1}$ gene was amplified from genomic DNA of *Serratia marcescens* strain AK9373 (7) and cloned into the expression vector pACSE3 (4) where it is expressed from the plasmid's pTAC promoter upon induction with 100 μM IPTG (isopropyl-β-D-thiogalactopyranoside). The sequence of the $bla_{IMP-1}$ allele in the resulting plasmid, pIMP1, was determined to be identical to the published sequence (1) (GenBank accession no. AF416297) except for a silent T-to-C mutation at bp 15 of the coding sequence. Sequencing of the original amplicon confirmed that the polymorphism is present in *S. marcescens* strain AK9373.

The IMP-1 gene of pIMP1 was mutagenized by amplification with the highly error-prone polymerase Mutazyme (Stratagene). The mutagenesis reaction yielded 9.85 μg of amplicon, or $1.2 \times 10^{13}$ mutant molecules. The mutagenized amplicon was digested with restriction endonucleases *Bsp*HI and *Sac*I, and in eight separate reactions, the digested amplicons were ligated to plasmid pACSE3 and transformed into *Escherichia coli* strain DH5αE ([F⁻ ϕ80d*lacZ*ΔM15 Δ(*lacZYA-argF*)U169 *endA1 recA1 hsdR17*($r^-$ $m^+$) *deoR thi-1 phoA supE44* λ⁻ *gyrA96 relA1* Gal⁻) by selecting for tetracycline resistance to produce eight libraries containing $9.8 \times 10^6 \pm 1.4 \times 10^6$ (mean ± standard error) insert-bearing transformants per library. Because each library contained $<10^{-6}$ molecules of the original pool of mutant molecules, the libraries were essentially independent samples of the pool of mutant molecules, and the probability that sibling molecules were present in different libraries is negligible. The $bla_{IMP-1}$ genes of plasmids extracted from 10 randomly chosen transformants were sequenced. There was an average of 1.2 mutations per gene.

In pIMP1, the $bla_{IMP-1}$ gene is expressed under control of the inducible pTAC promoter that is regulated by the plasmid-borne $lacI^q$ repressor. In the presence of 100 μM IPTG, pIMP1 conferred an imipenem MIC of 2 μg/ml on the host strain DH5αE compared with the imipenem MIC of 0.125 μg/ml for DH5αE carrying only the pACSE3 vector. That is the same level of resistance that has previously been reported for IMP-1 in *E. coli* (12).

Each of the eight libraries was expanded by growth overnight in L broth containing tetracycline (in 1 liter, 10 g of tryptone, 5 g of yeast extract, 10 g of NaCl, 1 g of glucose, 15 mg of tetracycline). Bottles containing 50 ml of L broth with 100 μM IPTG, with twofold serial dilutions of imipenem from 8 μg/ml down to 0.0625 μg/ml, were inoculated with $1.1 \times 10^8$ cells (10 times the largest library size) of the libraries. For a control, a similar imipenem dilution series was inoculated with $1.1 \times 10^8$ DH5αE carrying the unmutagenized plasmid pIMP1.

After 48 h of incubation at 37°C, the control series and each of the library series grew at imipenem concentrations of 2 μg/ml and below, but none grew at concentrations in excess of

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2 μg/ml. Plasmid was prepared from each of the cultures growing in the presence of 2 μg of imipenem per ml and was transformed into *E. coli* strain DH5αE by selection for tetracycline resistance. The resulting populations of transformants and *E. coli* DH5αE/pIMP1 after imipenem selection were used to measure the MIC of imipenem in Muller-Hinton broth (Difco) containing 100 μM IPTG using an inoculum of $10^5$ cells per ml by broth serial dilution as previously described (4). For seven of the eight mutant populations and for the control, the imipenem MIC was 2 μg/ml, and for one mutant population, the MIC was 1 μg/ml. Thus, neither the initial selections nor the MIC measurements provided any evidence of mutants that exhibited increased resistance to imipenem.

Because MICs can detect only twofold increases in resistance, a more sensitive disk diffusion test was used to detect small improvements in resistance. Forty cultures were grown overnight in L broth containing tetracycline. The cultures were grown from individual colonies of transformed library 8 after imipenem selection. For a control, 10 cultures were grown from individual colonies of *E. coli* DH5αE/pIMP1. One hundred microliters of each culture was spread on a Muller-Hinton broth plate containing 100 μM IPTG, a BBL antibiotic disk containing 10 μg of imipenem was placed onto the center of each plate, and after 24 h of incubation at 37°C, the zone of inhibition was measured. The diameters of the zones of inhibition were 17.2 ± 0.2 mm for pIMP1 and 17.1 ± 0.1 mm for cells carrying plasmids from library 8 after imipenem selection. The results of the disk diffusion test thus confirm that the mutants failed to confer increased resistance to imipenem, leading to the strong prediction that the IMP-1 metallo-β-lactamase does not have the potential to evolve increased activity against imipenem.

Confidence in that prediction is based on a simulation of the in vitro evolution process using the program In vitro Evolution Simulator (6, 11). The program simulates the random mutation of the input sequence and determines the fraction of possible single and double amino acid substitutions that are obtained in a library of a given size. It is important to consider the effects of only one or two independent amino acid substitution mutations, because in nature mutations almost always arise one at a time, and each mutation must be fixed into microbial populations by selection. The input sequence was the IMP-1 sequence, the mutation frequency was 1.2 mutations per molecule, and the fraction of possible single and double amino acid substitutions obtained was calculated separately for each library. The mean fractions per library were 0.897 ± 0.009 of the single amino acid substitutions and 0.670 ± 0.01 of the double amino acid substitutions (mean ± standard error). For the eight libraries taken together, the probability of having failed to screen any particular single amino acid substitution enzyme is $1.0 \times 10^{-8}$, and the probability of having failed to screen any particular double amino acid substitution enzyme is $1.3 \times 10^{-4}$. These results predict, with >99.9% confidence, that $bla_{IMP-1}$ will not evolve to confer increased resistance to imipenem. That prediction depends on the sensitivity with which we can detect increased resistance in the laboratory. I cannot eliminate the possibility that increased resistance, below the level of laboratory detection, could be selected in nature.

It is clear from this study that the risks associated with the presence of $bla_{IMP-1}$ do not include the risk of evolving increased activity against imipenem. This study, alone, is not sufficient to justify reconsideration of policies concerning the use of imipenem. In order to understand the risks posed by metallo-β-lactamases, it will be necessary to conduct similar studies on representative members of each of the three metallo-β-lactamase subfamilies and to include all clinically relevant carbapenems in those studies.

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# Exhibit 12

*New York Times* op-ed containing my views on teaching problems with Darwinian theory

The New York Times

FRIDAY, AUGUST 13, 1999



# Teach Evolution — And Ask Hard Questions

By Michael J. Behe

BETHLEHEM, Pa.

The debate leading the Kansas Board of Education to abolish the requirement for teaching evolution has about the same connection to reality as the play "Inherit the Wind" had to the actual Scopes trial. In both cases complex historical, scientific and philosophical issues gave way to the simplifying demands of the morality play. If the schoolchildren of Kansas and other states are to receive a good science education, however, then we'll have to forgo the fun of demonizing each other, take a deep breath and start making a few distinctions.

Regrettably, the action of the Kansas board makes that much more difficult. Not only are teachers there now discouraged from discussing evidence in support of Darwin's theory, results questioning it won't be heard either.

For example, let's look at three claims of evidence for Darwinian evolution often cited by high school textbooks. First, as the use of antibiotics has become common, mutant strains of resistant bacteria have become more common, threatening public health. Second, dark-colored variants of a certain moth species evaded predation by birds because their color matched the sooty tree trunks of industrial England. Third, the embryos of fish, amphibians, birds and mammals look virtually identical in an early stage of development, becoming different only at later stages.

A relevant distinction, however, is that only the first example is true. The second example is unsupported by current evidence, while the third is downright false. Although light- and dark-colored moths did vary in expected ways in some regions of England, elsewhere they didn't. Further, textbook photographs showing moths resting on tree trunks in the day, where birds supposedly ate them, run afoul of the fact that the moths are active at night and don't normally rest on tree trunks. After learning about the problems with this favorite Darwinian example, an evolutionary scientist wrote in the journal Nature that he felt the way he did as a boy when he learned there was no Santa Claus.

The story of the embryos is an object lesson in seeing what you want to see. Sketches of vertebrate embryos were first made in the late 19th century by Ernst Haeckel, an admirer of Darwin. In the intervening years, apparently nobody verified the accuracy of Haeckel's drawings. Prominent scientists declared in textbooks that the theory of evolution predicted, explained and was supported by the striking similarity of vertebrate embryos. And that is what generations of American students have learned.

Recently, however, an international team of scientists decided to check the drawings' reliability. They found that Haeckel had, well, taken liberties: the embryos are significantly different from each other. In Nature, the head of the research team observed that "it looks like it's turning out to be one of the most famous fakes in biology." What's more, the embryonic stages shown in the drawings are actually not the earliest ones. The earliest stages show much greater variation.

> Children need facts, not soothing simplifications.

If I were teaching a high school biology course, I certainly would want my students to understand Darwin's theory of evolution by natural selection, which explains antibiotic resistance and a lot of other things. I would want them to know the many similarities among organisms that are interpreted in terms of common descent, as well as to understand the laboratory experiments that show organisms changing in response to selective pressure.

But I would also want them to learn to make distinctions and ask tough questions. Questions we might discuss include these:

If it's so difficult to demonstrate that small changes in modern moths are the result of natural selection, how confident can we be that Darwinian selection drove large changes in the distant past? If supposedly identical embryos were touted as strong evidence for evolution, does the recent demonstration of variation in embryos now count as evidence against evolution? If some scientists relied for a century on an old, mistaken piece of data because they thought it supported the accepted theory, is it possible they might even now give short shrift to legitimate contrary data or interpretations?

Discussing questions like these would help students see that sometimes a theory actively shapes the way we think, and also that there are still exciting, unanswered questions in biology that may require fresh ideas.

It's a shame that Kansas students won't get to take part in such a discussion. We should make sure that the students of other states do.

Emotions run very deep on the subject of evolution, and while the morality play generally casts religious people as the ones who want to limit discussion, some scientists on the "rational" side could fit that role, too. But if we want our children to become educated citizens, we have to broaden discussion, not limit it.

Teach Darwin's elegant theory. But also discuss where it has real problems accounting for the data, where data are severely limited, where scientists might be engaged in wishful thinking and where alternative — even "heretical" — explanations are possible. □

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